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14. ABSTRACT
The purpose of this pre-clinical concept award project was to develop a safe and effective field-forward treatment to reduce neuronal loss after traumatic brain injury and thereby to enhance cognitive, sensory and motor recovery. Our primary objective was to synthesize and screen new progesterone pro-drug candidates for relative solubility that are more potent, stable and easily administered by immediate corporal injection than natural progesterone under battlefield conditions. We also sought to determine whether the prodrug would be as effective as progesterone in promoting neuroprotection after TBI. These objectives were accomplished.

15. SUBJECT TERMS
Progesterone, field-forward treatment, functional recovery, neuroprotection, traumatic brain injury

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Table of Contents

	Page
Introduction…….……..…..	**1**
Body……..	**1**
Key Research Accomplishments…….…….	**7**
Reportable Outcomes……	**7**
Conclusion……	**8**
References……	**8**
Appendices……	**9**

FINAL REPORT: PROGESTERONE IN THE FIELD-FORWARD TREATMENT OF TRAUMATIC BRAIN INJURY W81XWH-08-1-0184

INTRODUCTION

Pre-clinical and clinical research demonstrates that the hormone progesterone (PROG) is a potent neurosteroid that, acutely administered, can dramatically reduce cerebral edema, inflammation, tissue necrosis and programmed cell death. Although successful, the use of PROG in the recently completed Phase II trial had several requirements that would render treatment in far-forward battlefield conditions infeasible. Natural PROG (nPROG) is lipid-soluble, so it cannot be administered intravenously (IV)—the fastest way to get the neurosteroid to the damaged brain. Therefore nPROG must be delivered via a more suitable vehicle. For the clinical trial, the hormone was individually mixed in hospital with the carrier just before it was used, and this had to be done under temperature-controlled conditions. The mixing of nPROG with its intralipid carrier takes several hours and the resulting solution is not completely stable. Thus time and temperature requirements make nPROG difficult to use under far-forward battlefield conditions.

The purpose of this *pre-clinical concept award* project was to see whether it was feasible to develop a safe and effective field-forward treatment to reduce neuronal loss after TBI and thereby to enhance cognitive, sensory and motor recovery. Our primary objective was to synthesize and screen new PROG pro-drug (PD) candidates for relative solubility that would be more potent, stable and easily administered by immediate injection under battlefield conditions. We also sought to determine whether the PD would be as effective as PROG in promoting neuroprotection after TBI.

Natural PROG's impressive neuroprotective properties, coupled with its intrinsic formulation and pharmacokinetic challenges, present a tremendous opportunity for a novel molecule that maintains PROG's therapeutic potential while overcoming its liabilities. We prepared a set of new compounds to meet this challenge. The results of our work were published in J Med Chem. 2009 52(19):6012-23 (Appendix 1).

BODY

Analog design strategy

We designed a set of analogs specifically to maintain the neuroprotective properties of PROG while addressing its poor solubility. Fig. 1 is a graphic representation of the general design concept for these analogs. We attached a non-toxic, water solubilizing appendage to PROG and other related steroids through a vulnerable linker that is cleaved in vivo to release the active parent steroid. All the compounds we tested demonstrated sufficient water solubility to allow them to be prepared in aqueous-based

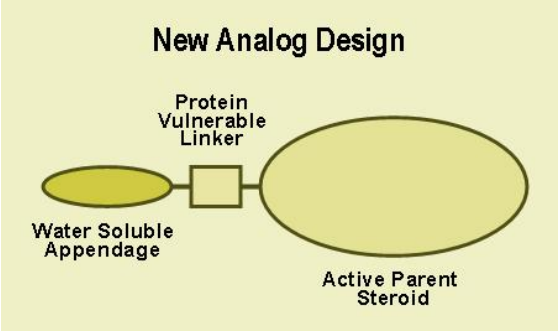


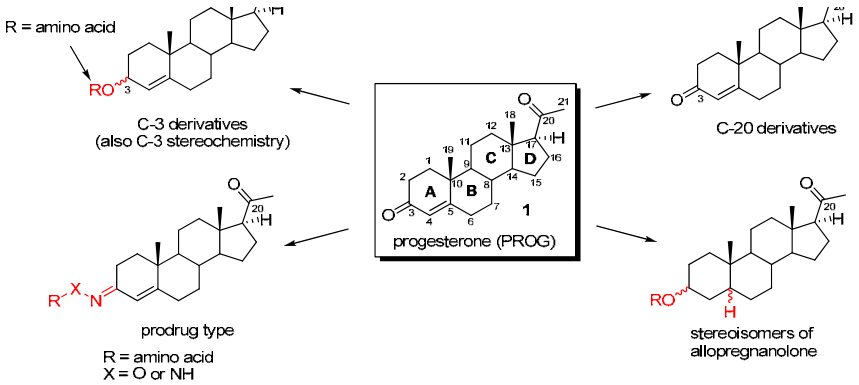
Figure 1. Schematic representation of the design strategy for novel compounds

formulations in the concentrations required for IV and intramuscular (IM) dosing.

Synthesis of PROG analogs

For specific details on synthesis please refer to MacNevin et al. (Appendix 1). Our strategy to enhance PROG water solubility was through the tethering of an amino acid to the hormone. Amino acids are non-toxic and provide a good degree of solubilizing capacity via the polarity of their amino group (Fig. 2). In addition, if the amino acid-PROG conjugate were to remain intact in vivo, the compound might be able to take advantage of amino acid active transport proteins that are known to be present within the blood brain barrier. Derivitization of PROG at either the carbon-3 (C-3) or C-20 carbonyl was conducted in order to examine the relative effect of such a selective modification. A PD would then, by definition, release PROG in vivo.

Figure 2. Overview of approach to the development of progesterone analogs



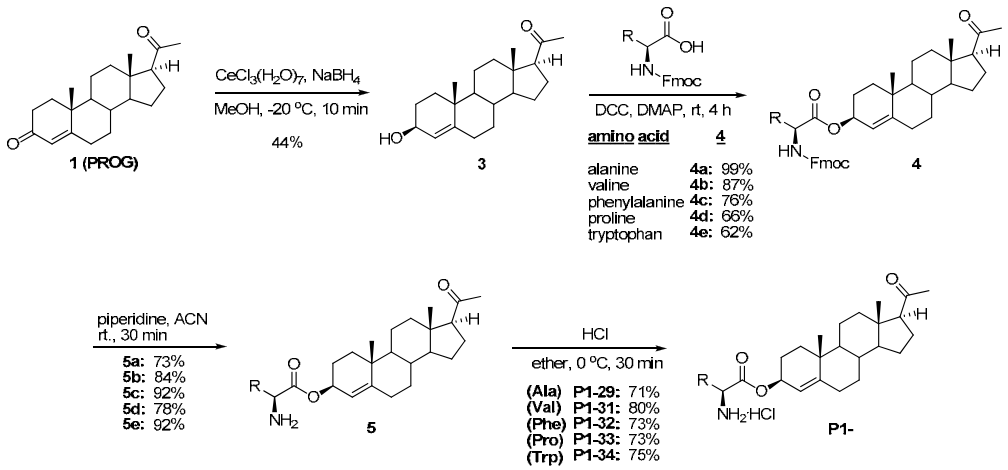
C-3 and C-20 PROG Derivatives

Selective reduction of the allylic C-3 carbonyl of PROG was achieved via the method of Luche using cerium trichloride heptahydrate in methanol (Scheme 1). The reaction was run at low temperature and terminated after only 10 min to avoid additional over-reduction of the more sterically hindered C-20 carbonyl. The C-3 reduction product 3 was then coupled with a series of different N-Fmoc protected amino acids under standard acid-alcohol coupling conditions using N,N'- dicyclohexylcarbodiimide (DCC) in dichloromethane (DCM) with a catalytic amount of 4-di(methylamino)pyridine (DMAP) to give the ester substrates 4a – 4e. Following ester formation, the Fmoc protecting group was easily removed by treatment with an excess of piperidine in acetonitrile (ACN) to give the free amines 5a – 5e, which were then prepared as their respective hydrochloride (HCl) salts.

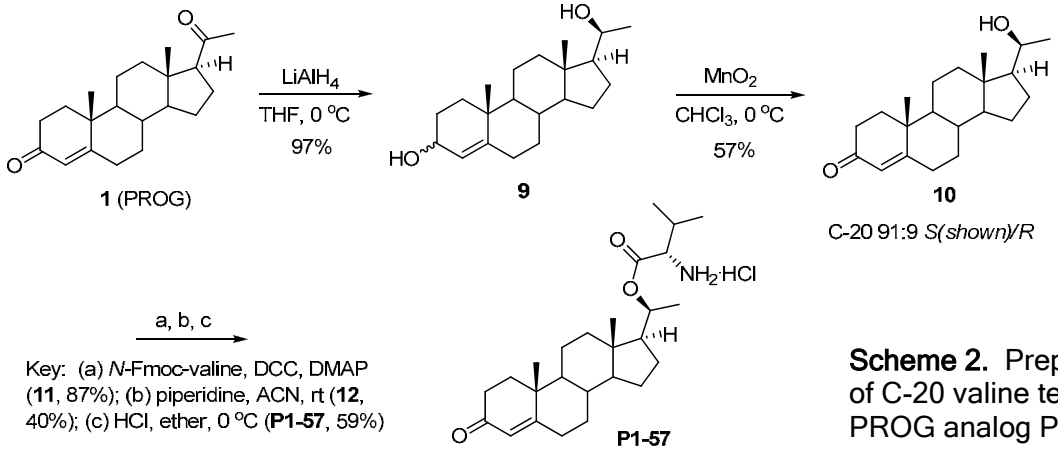
Preparation of the C-20 amino acid tethered amino acid analog P1-57 began with a total reduction of PROG using lithium aluminum hydride (LAH) to give the di-hydroxyl compound 9 (Scheme 2). Allylic oxidation of 9 with MnO_2 gave the C-20 mono-hydroxy derivative 10, which was then coupled with L-valine through the same series of steps as had proven successful for the C-3 tethered series of analogs.

PROG-Oxime Derivatives

One strategy we used in developing a PROG PD was to insert an oxime linker between the PROG scaffold and the solubilizing amino acid. Regioselective oximation of PROG was desired to minimize alteration of the molecule and to keep the final molecular weight of the compound within the limit of 500. A selective method for the formation of a ketal at C-20 was found through application of a procedure



Scheme 1. Preparation of C-3 amino acid tethered PROG analogs.



Scheme 2. Preparation of C-20 valine tethered PROG analog P1-57

developed for C-9 ketalization of the Wieland-Miescher ketone, which utilizes ethylene glycol as solvent and a stoichiometric amount of p-toluene-sulfonic acid (Scheme 3). The protected steroid 26 was then added to hydroxylamine to afford a 2:1 E/Z mixture of oximes 27 and 28 that were separable by conventional chromatography and isolated.

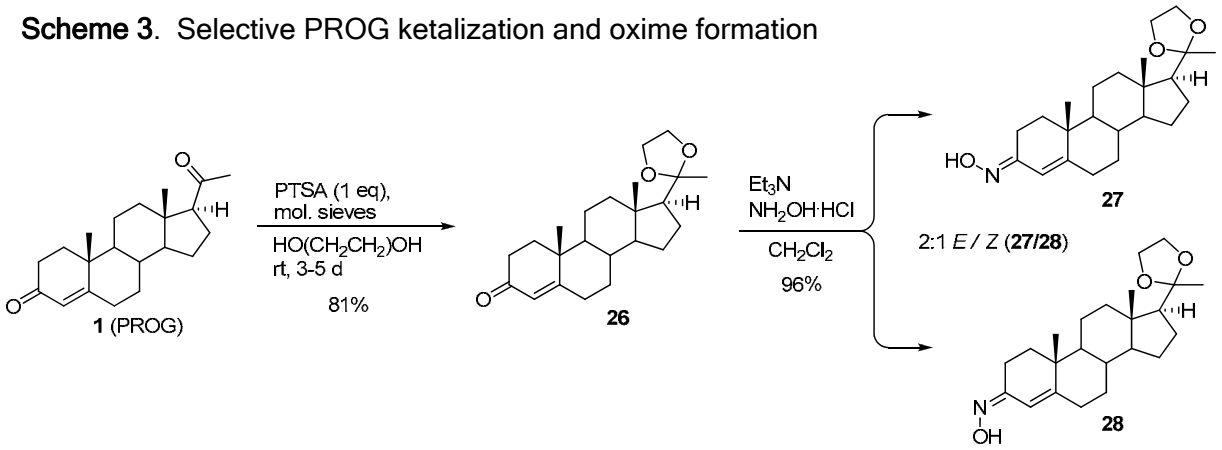
The oxime products 27 and 28 were each then coupled to N-Fmoc protected L-valine (products 29 and 31, Scheme 4). We then modified the reaction procedure to incorporate a low temperature workup and rapid conversion of the free amine products into their HCl salt. This served to eliminate the unintended cleavage of the amino acid side chain. Both the E oxime PD compound P1-185 and the Z oxime derivative P1-186 were isolated as white solids in good yield.

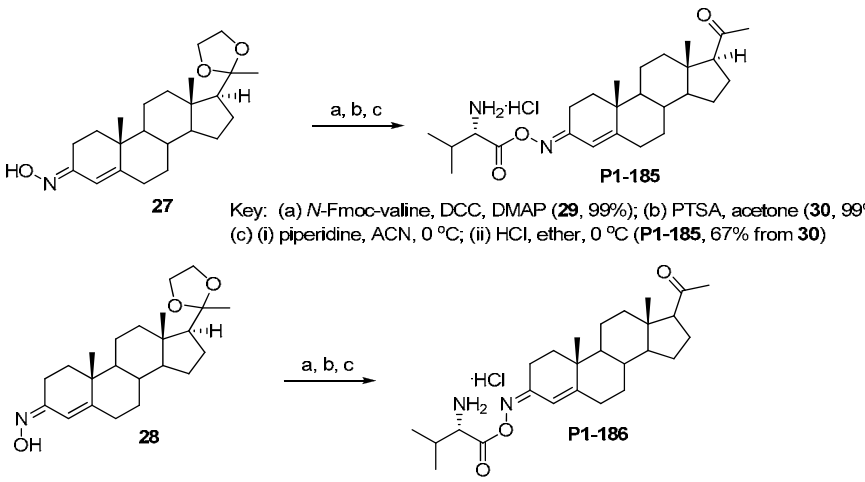
Solubility and Biological Testing Data

Solubility Data

We have now screened several products for solubility (Fig. 3). Solubility testing was done in phosphate buffered saline. Compounds were added in small portions at room temperature with stirring until a visible endpoint of saturation was reached. Neither nPROG nor allopregnanolone (ALLO) showed any degree of solubility by this method (designated at < 0.05 mg/mL solubility). The valine coupled C-3-β-hydroxy PROG derivative P1-31 showed the best solubility (3.85 mg/mL) of the different amino acid substituted

Scheme 3. Selective PROG ketalization and oxime formation





Scheme 4. Preparation of oxime-based PROG prodrug compounds P1-185 and P1-186

analogs within the series. Compound P1-133, the 3α-5β ALLO isomer, showed the highest solubility within the group, though this was still fairly low (0.33 mg/mL). *Both the E and Z oxime derivatives P1-185 and P1-186 however showed excellent solubility, with values of 13.0 and 15.2 mg/mL respectively.* These values far surpass the desired target of at least 1.0 mg/mL which would allow for facile compound formulation in an aqueous based solution.

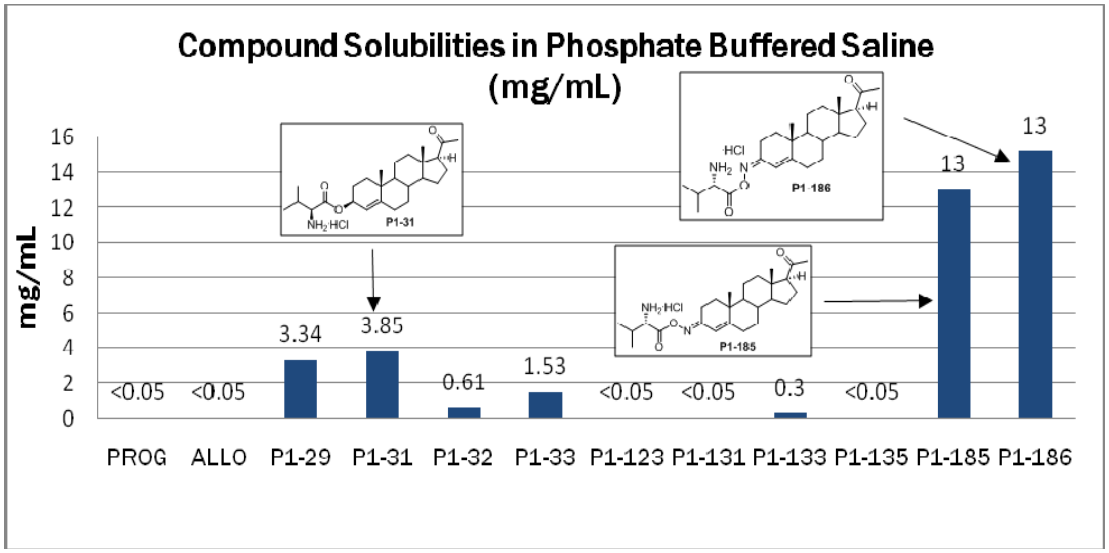


Figure 3. PROG analog solubilities in phosphate buffered saline

In Vitro Assay Data

Primary cortical cells were seeded in multi-well plates and cultured for 8-10 days in neurobasal medium. These cells were then pre-treated with various concentrations (doses) of the different PROG analogs (0.1, 1, 5, 10, 20, 40, and 80 µM) for 24h. Cells were then exposed to glutamate (0.5µM) for 24h and cytotoxicity was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. When the concentration of a given test compound is not considered, both PROG and ALLO showed greater protection against cell death than the analogs (Table 2). However, several of the derivatives proved to be significantly more potent than PROG or ALLO when all compounds were compared at the 5 µM concentration. The P1-185 and P1-186 oxime PD compounds produced the highest levels of cell survival among the new compounds we have screened to date. The C-20 reduced PROG derivative P1-57 and the ent-PROG derivative P2-13 also showed significant reductions in cell death. Interestingly, the latter showed much better protection against glutamate induced cytotoxicity than the comparable nPROG C-3 hydroxyl derivative P1-31 (26% versus 8%).

Table 2. Reduction in glutamate-induced excitotoxic cell death in primary cortical neurons as evaluated by MTT assay. Rat primary cortical cells (E18) were pre-treated with different concentrations of drugs for 24h and subsequently exposed to glutamate (0.5µM) for 24h. Test compounds were present in the culture medium during glutamate exposure and were dissolved in DMSO. Glutamate was dissolved in PBS (pH 7.4). The final concentration of DMSO was <5 µl/ml medium.

TABLE 2		
compound	reduction in cell death best con-centration (%)	reduction in cell death at 5 µM (%)
PROG	42 (20 µM)	4
ALLO	40 (80 µM)	-3
P1-29	13 (5 µM)	13
P1-31	8 (5 µM)	8
P1-33	15 (5 µM)	15
P1-34	1 (5 µM)	1
P1-57	30 (10 µM)	23
P1-185	27 (5 µM)	27
P1-186	34 (5 µM)	34
P2-13	26 (5 µM)	26

In Vivo Cerebral Edema Assay Data

To test for efficacy and safety we used a well-established animal model of TBI. This approach enabled us to investigate the efficacy of the PROG analog compounds relative to nPROG in reducing cerebral edema following injury (Fig. 4). Sham controls did not receive injury and served as a control group for possible anesthesia and stress factors. The vehicle group received bilateral cortical injury but were given only the drug carrier (22.5% 2-hydroxypropyl-β-cyclodextrine in water).

Several of the analogs showed equivalent efficacy to PROG in the cerebral edema assay, including the valine tethered C-3-β-hydroxy PROG derivative P1-31 and the oxime based PD compound P1-185. Compound P1-131, the valine coupled derivative of ALLO itself, showed the greatest edema reduction among the ALLO isomer group. Perhaps most notable however, was the activity of oxime PD P1-186, which showed an average reduction in edema levels almost twice that of PROG.

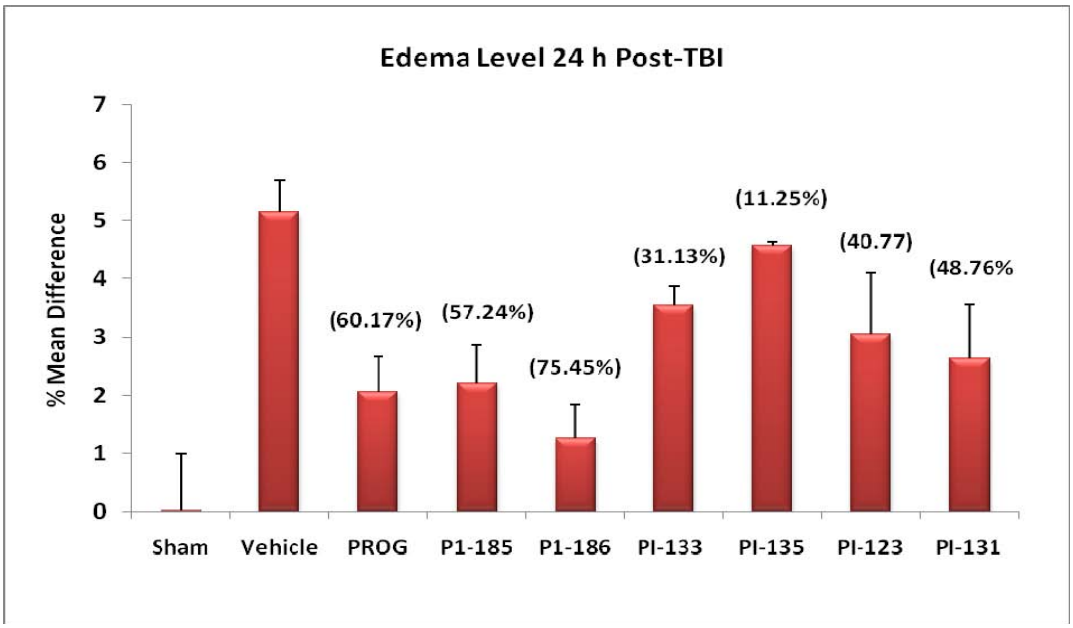


Figure 4. Effect of various analogs/pro-drugs on edema level 24h post-TBI. Values are expressed as mean ±SEM. Test compounds (8mg/kg) were administered twice: first dose (IP) injections 1h post-TBI, second dose at 6h (SC) post-TBI. At 24h, animals were sacrificed and brain sections removed for edema assay. Values in parentheses represent percent decrease in edema level compared to vehicle.

Metabolic profile of PROG PD PI-185 in rats

We have shown in rats that PI-185 is converted in vivo to PROG (Fig. 5). In this study, rats (n=3) were dosed IV and IP at 10 mg/kg. The plasma profiles after IV dosing (Fig. 5a) suggested that the conjugate (PI-185) is rapidly converted to an intermediate metabolite where the water soluble appendage is cleaved, but the linker remains bound to PROG. This intermediate rapidly builds significant plasma levels and is then slowly converted to PROG, which is in turn produced with a Cmax of >200 ng/mL. A similar profile was observed after IP administration (Fig. 5b), suggesting that the intermediate metabolite survives first

pass metabolism, but PI-185 does not. This is an encouraging demon-stration of PD behavior that could extend the beneficial effects of treatment over time.

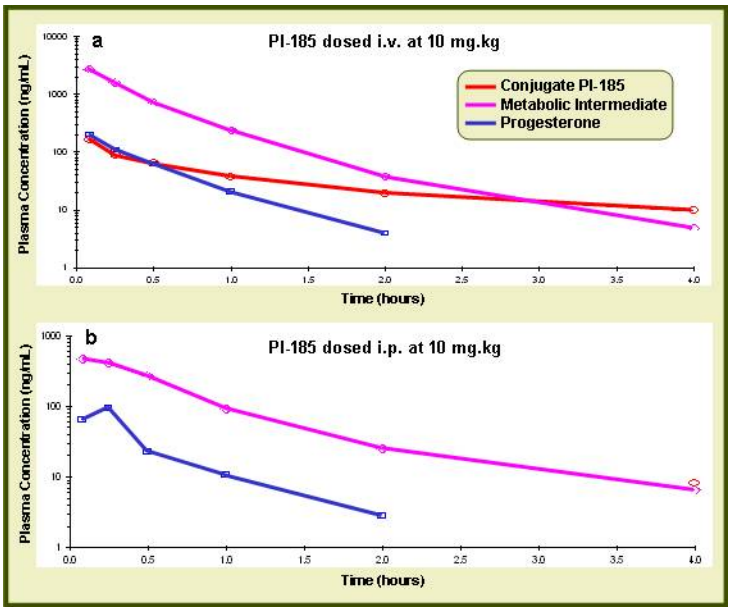


Figure 5. Plasma concentration profile of progesterone conjugate PI-185 and its metabolites in rats

RESEARCH ACCOMPLISHMENTS

1. Proved the feasibility of developing a set of water-soluble PD analogs of PROG that are easier to administer in field conditions compared to PROG in lipid formulation.

2. Demonstrated that the PDs possess many of the functional characteristics of native PROG.

3. Tested the PD derivatives against PROG itself in animals with bilateral brain injuries and found that these agents were easier to administer, had more rapid absorption, metabolize to PROG, and can reduce cerebral edema as well as native PROG or its metabolite, ALLO.

REPORTABLE OUTCOMES (See also Appendices)

- **Manuscript**: "Development and screening of water-soluble analogs of progesterone and allopregnanolone in models of brain injury." MacNevin CJ, Atif F, Sayeed I, Stein DG, Liotta DC. J Med Chem. 2009 Oct 8;52(19):6012-23.
- **Society for Neuroscience poster presentation**: Development and screening of water soluble analogs of progesterone: Potential for an innovative, safe and effective approach to acute traumatic brain injury treatment. Iqbal Sayeed, Christopher J. MacNevin, Fahim Atif, Dennis C. Liotta, Donald G. Stein
- **Presentation**: "Development and screening of water soluble analogues of progesterone: Potential for an innovative, safe and effective innovative approach to acute traumatic

brain injury treatment." Military Health Research Forum, Kansas City MO, August 31-September 3, 2009

- **Patent application:** Steroid analogs for neuroprotection, US Provisional Patent Application Nos. 61/032,315, 61/031,629, and 61/031,567
- **New Pre-proposal**: "Water soluble progesterone derivative in the far-forward treatment of TBI" (DM102619) submitted to DOD December 18, 2009

CONCLUSION

- Several novel analogs of PROG, its natural metabolite ALLO, and the enantiomer of PROG were synthesized and screened for solubility and for their potential as neuroprotective agents.
- The use of an amino acid tether was shown to be an effective method for greatly enhancing the solubility of PROG and other related steroidal compounds.
- Several compounds have shown nearly equivalent activity to PROG and ALLO in an in vitro assay designed to assess their ability to enhance neuronal cell survival.
- The C-3-β-hydroxy derivative P1-31 and the oxime derived compound P1-185 both showed equivalent capacity relative to PROG for reducing cerebral edema following cranial injury in a whole animal model of TBI.
- Based on solubility data we were able to identify two potent candidates: P1-185 (13 mg/ml) and P1-186 (15.6 mg/ml). Both showed much better solubility in water than PROG (<0.05 mg/ml).
- PK studies revealed that the oxime-based compound P1-186 generated PROG in vivo when given IV at 10 mg/kg. Maximum serum levels of 100 ng/ml were attained over the course of 12h.
- *In vitro* data demonstrated that both analogs were more effective in reducing glutamate-induced neuronal loss compared to PROG at similar (5μM) dose.

Conclusion: There are currently no clinically effective and available acute-stage treatments for traumatic brain injuries. PROG, a natural hormone, is now in Phase III testing as a treatment for moderate to severe TBI, but the current formulation has to be administered in hospital under controlled conditions. The use of a more highly water soluble PD of PROG will be useful for delivering PROG to brain-injured subjects under emergency conditions and achieving neuroprotective effects. This could represent a breakthrough step in the early treatment of TBI that will result in less mortality and better functional outcomes for wounded warriors.

REFERENCES

Macnevin C J, Atif F, Sayeed I, Stein D G and Liotta D C (2009) Development and screening of water-soluble analogues of progesterone and allopregnanolone in models of brain injury. *J Med Chem* 52, 6012-23.

APPENDICES

Appendix 1: MacNevin CJ, Atif F, Sayeed I, Stein DG, Liotta DC, "Development and screening of water-soluble analogs of progesterone and allopregnanolone in models of brain injury." J Med Chem. 2009 Oct 8;52(19):6012-23.

Appendix 2: Sayeed, I., MacNevin, C.J., Atif, F., Liotta, D.C., Stein, D.G., Development and screening of water soluble analogs of progesterone: Potential for an innovative, safe and effective approach to acute traumatic brain injury treatment. Society for Neuroscience Annual Meeting, Oct 19, 2009, poster 335.4/K24.

Appendix 3: Sayeed I, MacNevin CJ, Atif F, Nachus MG, Liotta DC, Stein DG. Development and screening of water soluble analogues of progesterone: Potential for an innovative, safe and effective innovative approach to acute traumatic brain injury treatment. Military Health Research Forum, Kansas City MO, August 31-September 3, 2009.

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Appendix 5: Pre-proposal, **"**Water soluble progesterone derivative in the far-forward treatment of TBI," (DM102619) submitted to DOD December 18, 2009. PI, Donald G. Stein, Ph.D.

APPENDIX 1: MacNevin et al.

6012 J. Med. Chem. 2009, 52, 6012–6023
DOI: 10.1021/jm900712n

Journal of Medicinal Chemistry Article

Development and Screening of Water-Soluble Analogues of Progesterone and Allopregnanolone in Models of Brain Injury

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Preclinical and clinical research findings have revealed that the hormone progesterone, when acutely administered, can dramatically reduce cerebral edema, inflammation, tissue necrosis, and programmed cell death following traumatic brain injury (TBI). The poor aqueous solubility of progesterone, however, limits its potential use as a therapeutic. Several chemically novel analogues of progesterone and its natural metabolite allopregnanolone have been synthesized and screened using both in vitro and whole animal models of TBI. The new derivatives demonstrated greatly improved solubility and select compounds have shown equivalent effectiveness to progesterone in reducing cerebral edema after TBI.

Introduction

Traumatic brain injury (TBI[a]) is a significant public health problem that affects nearly 1.5 million Americans each year, resulting in approximately 235000 hospitalizations, 80000 cases of long-term disability, and 50000 deaths.[1] An estimated 5.3 million Americans currently require long-term assistance in performing basic activities of daily living as the result of having suffered a TBI,[2] and care related costs for the treatment of TBI patients has been estimated to total nearly $60 billion annually.[3] In addition, a recent comprehensive study of Iraq and Afghanistan war veterans reports that 19% of those surveyed had suffered a TBI, making it one of the so-called "signature injuries" found among soldiers returning from duty.[4]

Despite several decades of effort from the scientific community, no single pharmacological agent or treatment protocol has been found that results in consistently improved outcomes following TBI.[5] A recent meta-analysis of studies evaluating the effectiveness of five experimental treatments for TBI (hyperventilation, mannitol, cerebrospinal fluid drainage, barbiturates, and corticosteroids) showed that none of the interventions reliably reduced death or disability.[6] The Corticosteroids After Significant Head Injury (CRASH) trial, which originally sought to utilize a population group of 20000, was terminated at just over 10000 patients as it became clear that the treatment group (0.4 g methylprednisolone per h for 48 h) had a higher mortality rate than that of the control group.[7] Hypothermia has shown some favorable effects among brain injured patients.[8] However, clinical results using hypothermia have been variable and the treatment is thought to be potentially harmful to patients over the age of 45.[9]

A growing body of evidence indicates that progesterone (**1**, Scheme 1) may be a promising alternative therapeutic candidate for the treatment of TBI.[10] Progesterone is an endogenous steroid produced by the adrenal glands and the corpus luteum as well as by neurons and glial cells within the central and peripheral nervous systems.[11] Just as progesterone has been associated with a variety of protective roles during gestation, it has also been linked to several different but mutually supportive modes of neuroprotection following TBI, including the attenuation of cerebral edema,[12] the reduction of lipid peroxidation and oxidative stress,[13] anti-inflammatory effects,[14] reduction of cellular apoptosis,[15] inhibition of excitotoxicity,[16] and assisting in the repair of myelin.[17]

In light of the promising preclinical evidence, a phase II, randomized, placebo-controlled human clinical trial was initiated in order to evaluate the safety and potential efficacy of intravenous progesterone administration for the treatment of acute TBI.[18] The study concluded that administration of progesterone to brain-injured patients was safe based on the finding that no serious adverse events were noted and that the rates of adverse events among treatment and placebo groups were similar. The most significant result from the study however was that *the rate of mortality among severely injured patients treated with progesterone was reduced by over 60% relative to the placebo group*. In addition, patients in the moderate group showed significantly better functional outcomes at 30 days postinjury. A second single-center trial with severely brain-injured patients in which outcomes were studied over a 6 month period supported the results of the first study.[19]

Despite the encouraging patient outcomes observed in these clinical trials, the eventual use of progesterone as a therapeutic is limited due to its poor aqueous solubility. For human use, the hormone had to be individually mixed with the vehicle solution for several hours under temperature controlled conditions. The resulting formulation is not stable at room temperature for long periods of time and therefore had to be

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[a] Abbreviations: TBI, traumatic brain injury; Fmoc, 9-fluorenylmethyl; HCl, hydrochloride; PTSA, *p*-toluenesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IP, intraperitoneal; SC, subcutaneous; RBF, round-bottom flask.

 Published on Web 09/08/2009

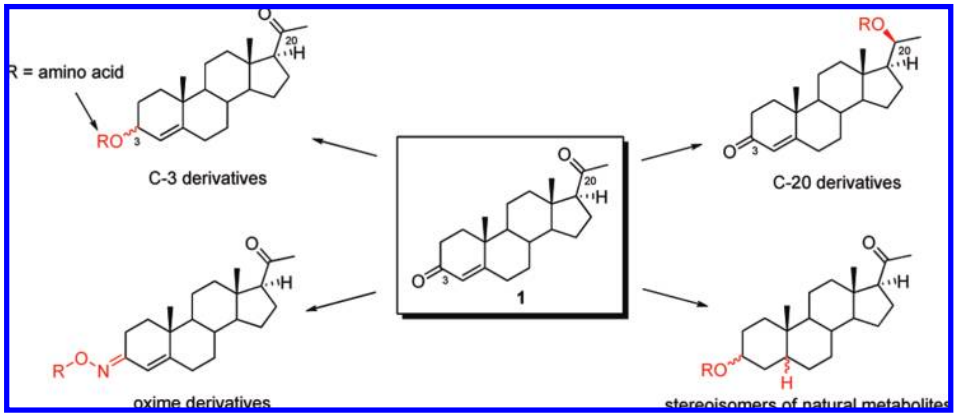
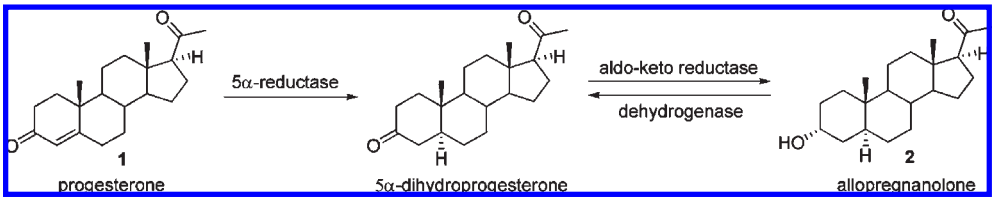
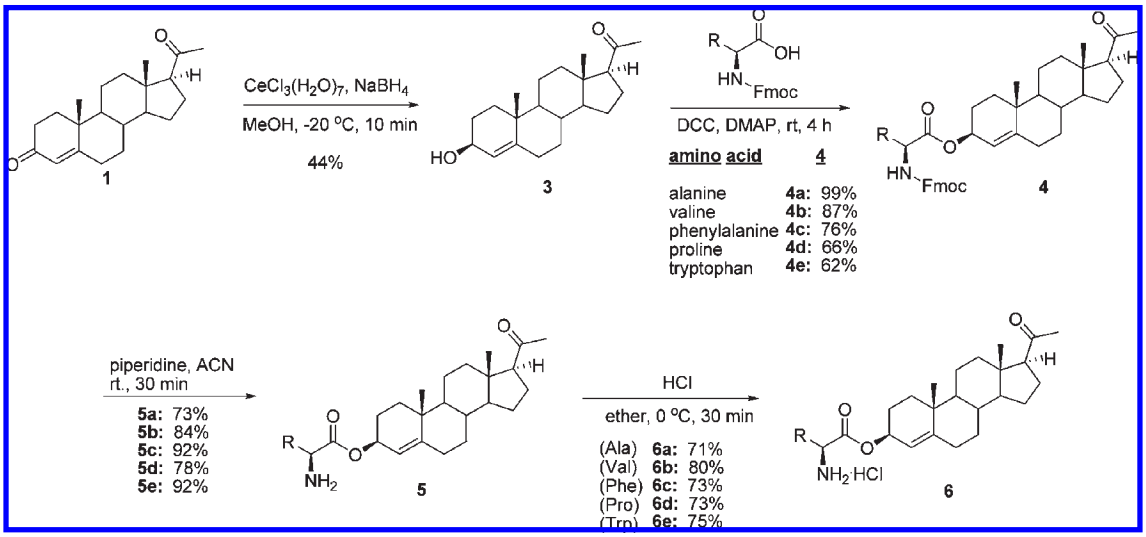


Figure 1. Overview of approach to the development of progesterone analogues.

Scheme 1. Enzymatic Conversion of Progesterone (**1**) to Allopregnanolone (**2**)



Scheme 2. Preparation of C-3 Amino Acid Tethered Progesterone (**1**) Analogues



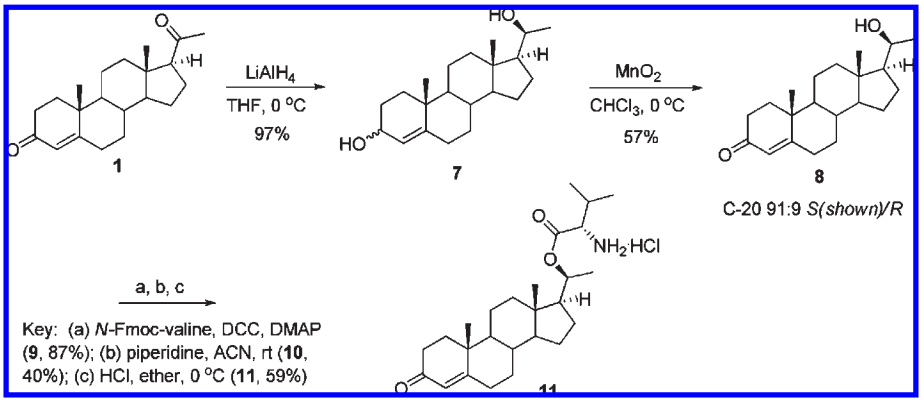
used quickly following its preparation. The most potentially beneficial application of progesterone for traumatic brain injury would be to give it as soon as possible in an emergency situation, and thus a lengthy preparation time for the treatment would compromise some of its beneficial effects in reducing the injury cascade. Access to a more highly water-soluble progesterone analogue that would be amenable to preformulation and long-term storage was therefore pursued.

Results and Discussion

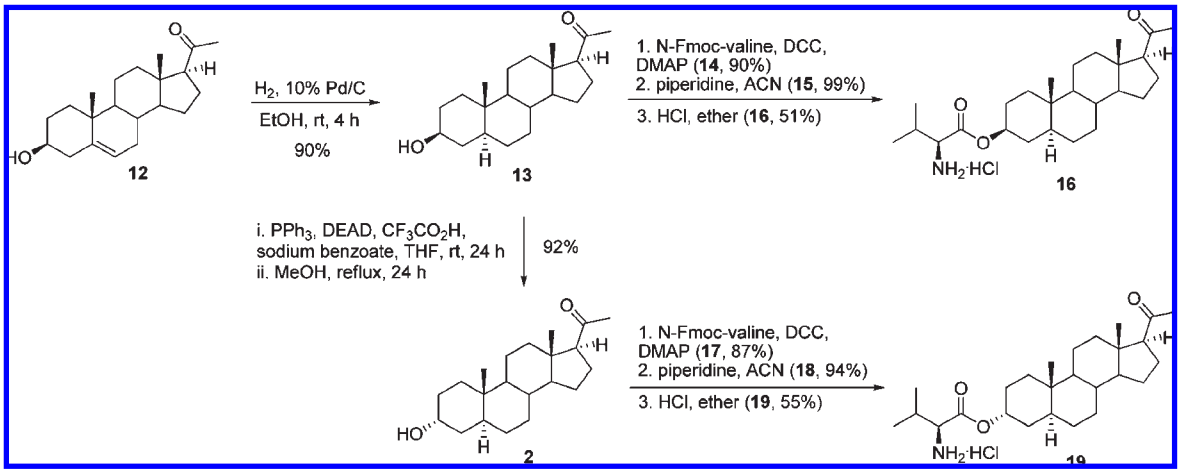
Chemistry. An approach to the development of novel progesterone derived compounds was designed in order to explore structure−activity relationships potentially arising from several different sources (Figure 1). The fundamental strategy chosen to enhance progesterone's water solubility was through the tethering of an amino acid to the compound. Derivatization of **1** at either the carbon-3 (C-3) or carbon-20 (C-20) carbonyl was planned in order to examine the relative effect of such selective modification. The progesterone metabolite allopregnanolone (**2**, Scheme 1) has shown many of the same neuroprotective benefits afforded by progesterone,[20] therefore the development of water-soluble allopregnanolone derivatives was an additional goal of interest. One final aim of particular priority was to develop a water-soluble progesterone prodrug. This was thought to be potentially achievable through the selective incorporation of an oxime tether between steroid and amino acid components.

C-3 and C-20 Progesterone Derivatives. Selective reduction of the allylic C-3 carbonyl of progesterone (**1**) was achieved via the method of Luche (Scheme 2).[21] The reaction was run at low temperature and terminated after only 10 min in order to avoid additional over-reduction of the more sterically

Scheme 3. Preparation of C-20 Valine Tethered Progesterone Analogue **11**



Scheme 4. Preparation of 5α Allopregnanolone Isomer Derivatives **16** and **19**



hindered C-20 carbonyl. The C-3 reduction product **3** was then coupled with a series of different *N*-9-fluorenylmethyl (Fmoc) protected amino acids to give substrates **4a**−**4e**. The Fmoc protecting group was subsequently removed to give the free amines **5a**−**5e**, which were then prepared as their respective hydrochloride (HCl) salt (**6a**−**6e**).

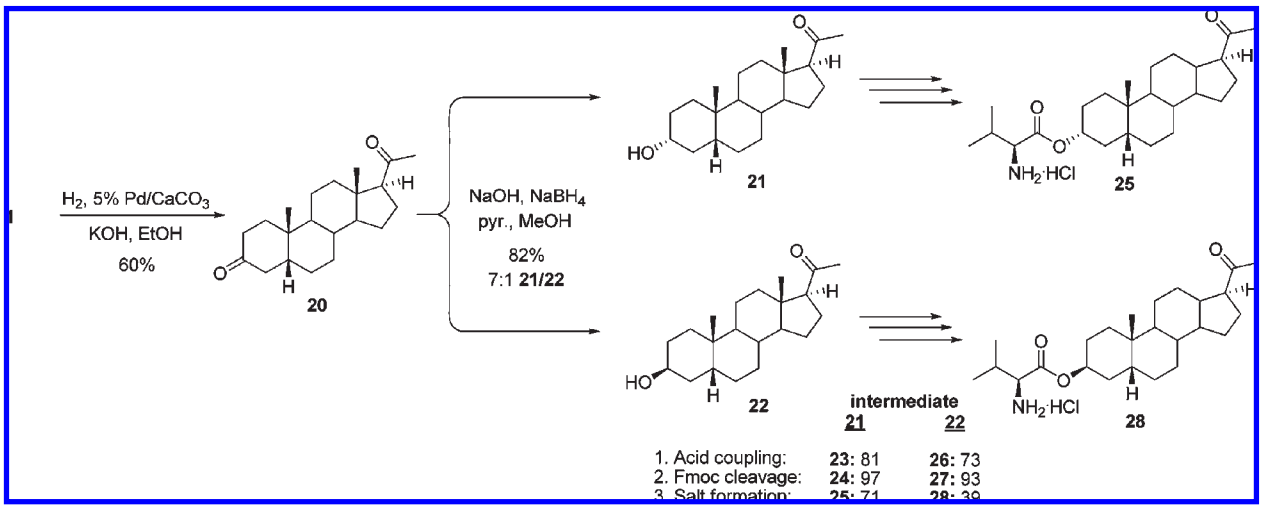
Preparation of the C-20 amino acid tethered progesterone analogue **11** began with a total reduction of **1** to give the dihydroxyl compound **7** (Scheme 3). Allylic oxidation of **7** gave the C-20 monohydroxy derivative **8**, which was then carried forward to generate the L-valine coupled hydrochloride salt **11** through the same series of steps as had proven successful for the C-3 derivatives.

Allopregnanolone Derivatives. Synthesis of epiallopregnanolone (**13**, Scheme 4) was achieved by catalytic reduction of the commercially available steroid pregnenolone (**12**).[22] Amino acid coupling, deprotection, and salt formation proceeded as with previous analogues to give the 3β-5α allopregnanolone isomer derivative **16**. Mitsunobu inversion of the 3β-hydroxy center of **13** gave allopregnanolone (**2**) in good yield.[23] Compound **2** was then prepared as its valine coupled HCl salt to give the 3α−5α compound **19**.

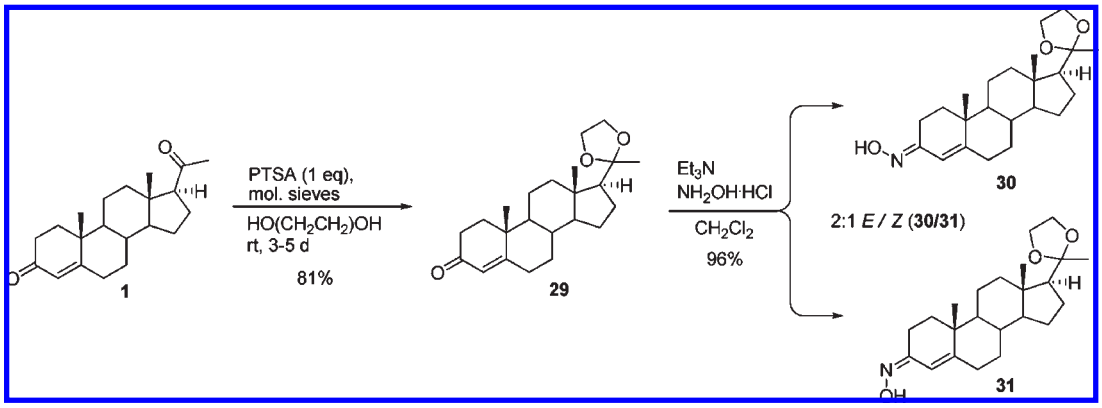
The two 5β allopregnanolone isomers were prepared from progesterone (Scheme 5). Hydrogenation of **1** in the presence of hydroxides is known to provide the 5β reduction product (**20**).[24] Treatment of **20** with $NaBH_4$ in methanol led to selective reduction of the C-3 carbonyl in the presence of the C-20 ketone.[25] The reduction was selective for C-3 but gave a 7:1 mixture of 3α-hydroxy (**21**) to 3β-hydroxy (**22**) products. These compounds were separated by conventional column chromatography and carried forward as in previous series to give the 3α−5β derivative **25** and 3β−5β derivative **28**.

Oxime Derivatives of Progesterone. One strategy taken toward the development of a progesterone prodrug was to utilize an oxime linker between the progesterone scaffold and the solubilizing amino acid. Regioselective oximation of progesterone was desired in order to both minimize alteration to the molecule and to keep the final molecular weight of the compound within the range associated with the majority of drug-like molecules.[26] A selective method for the formation of a ketal at C-20 was found through application of a procedure developed for C-9 ketalization of the Wieland−Miescher ketone,[27] which utilizes ethylene glycol as solvent and a stoichiometric amount of *p*-toluenesulfonic acid (PTSA). The reaction with **1** required more time (3−5 d) than for the Wieland−Miescher ketone but nevertheless achieved exclusive ketalization at the C-20 position (Scheme 6). The protected steroid **29** was then added to hydroxylamine to afford a 2:1 *E*/*Z* mixture of oximes **30** and **31** that were separable by conventional chromatography and isolated in excellent yield. Assignment of the compounds as their respective isomer was based on characteristic polarity and ^{1}H and ^{13}C chemical shifts relative to previously reported oxime derivatives of progesterone.[28]

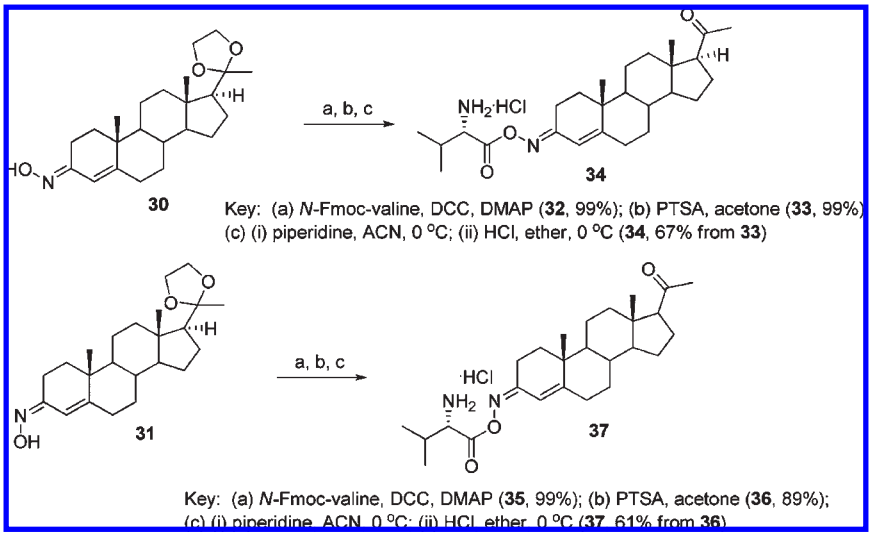
Scheme 5. Preparation of 5β Allopregnanolone Isomer Derivatives **25** and **28**



Scheme 6. Selective Progesterone Ketalization and Oxime Formation



Scheme 7. Preparation of Oxime Derivatives of Progesterone, Compounds **34** and **37**



The oxime products **30** and **31** were each then coupled to *N*-Fmoc-L-valine (**32** and **35**, Scheme 7). The ketal was found to be easily removed at this point through treatment with PTSA in acetone. The free amine products resulting from subsequent Fmoc cleavage were however found to be unstable when concentrated at room temperature for any length of time. The reaction procedure was therefore modified to incorporate a low temperature workup and rapid conversion of the free amine products into their HCl salt. These procedural changes served to eliminate the unintended

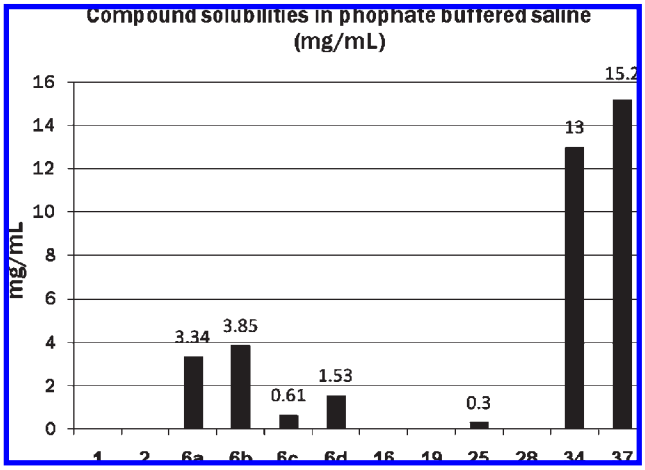


Figure 2. Progesterone analogue solubilities in phosphate buffered saline. Compounds with no value given showed less than 0.05 mg/mL solubility by this method.

cleavage of the amino acid side chain. Both the *E* oxime prodrug compound **34** and the *Z* oxime derivative **37** were isolated as white solids in good yield.

Solubility and Biological Testing Data. Solubility Data. Several compounds were screened for solubility (Figure 2). Compounds were added to phosphate buffered saline in small portions at room temperature with stirring until a visible end point of saturation was reached. Neither progesterone (**1**) nor allopregnanolone (**2**) showed any measurable degree of solubility by this method. The valine coupled 3β-hydroxy progesterone derivative **6b** showed the best solubility (3.85 mg/mL) of the different amino acid substituted analogues within that series. Compound **25**, the 3α−5β allopregnanolone isomer, showed the highest solubility within that group, although this was still fairly low (0.33 mg/mL). Both the *E* and *Z* oxime derivatives **34** and **37** showed excellent solubility, with values of 13.0 and 15.2 mg/mL, respectively. These values far surpass the desired target of at least 1.0 mg/mL, which would allow for facile compound formulation in an aqueous based solution.

In Vitro Assay Data. Primary cortical cells were seeded in multiwell plates and cultured for 8−10 days in neurobasal medium. Cells were then pretreated with various concentrations of a different progesterone analogue (0.1, 1, 5, 10, 20, 40, and 80 μM) for 24 h. Cells were next exposed to glutamate (0.5 μM) for the following 24 h and cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. When the concentration of a given test compound is not considered, both progesterone (**1**) and allopregnanolone (**2**) showed greater protection against cell death than any of their analogues (Table 1). However, the concentrations of these compounds required to achieve their most significant effect were relatively high (20 μM for **1**, 80 μM for **2**). When all compounds are compared at the 5 μM concentration, several of the new derivatives showed significant reduction in cell death, whereas the parent compounds **1** and **2** did not. Treatment of cells with the oxime derivative compounds **34** and **37** achieved the highest levels of cell survival at the 5 μM concentration among the new compounds screened.

In Vivo Cerebral Edema Assay Data. Several studies have demonstrated that the exogenous administration of progesterone reduces cerebral edema in both male and female rats after bilateral cortical contusions of the medial frontal cortex.[10c,10e,10f,12e] A similar whole animal model of TBI (bilateral cortical contusions of the medial frontal cortex) was employed in order to investigate the potential efficacy of the progesterone analogue compounds relative to progesterone in reducing cerebral edema following injury (Figure 3). The sham animals did not receive an injury but served as a control group for possible anesthesia and stress factors. The vehicle group was subjected to cortical injury but received only the drug carrier (22.5% 2-hydroxypropyl-β-cyclodextrine in water). The initial dose of progesterone and analogues was given at 1 h postinjury, delivered by intraperitoneal (IP) injection for rapid absorption. Subsequent injections at 6 h postinjury were given subcutaneously (SC). The treatment protocol was based on previous studies in our laboratory.[10g] Several of the analogues showed equivalent efficacy to progesterone (**1**) in the cerebral edema assay, including the valine tethered 3β-hydroxy progesterone derivative **6b** and the oxime based prodrug compound **34**. Compound **19**, the valine coupled derivative

Table 1. Reduction in Glutamate-Induced Excitotoxic Cell Death in Primary Cortical Neuron as Evaluated by MTT Assay[a]

compd	reduction in cell death (best concentration) (%)	reduction in cell death at 5 μM (%)
1	42 ± 2.9* (20 μM)	4 ± 2.5
2	40 ± 1.8* (80 μM)	−3 ± 4.7
6a	13 ± 0.84* (5 μM)	13 ± 0.84*
6b	8 ± 3.4 (5 μM)	8 ± 3.4
6d	15 ± 1.5* (5 μM)	15 ± 1.5*
6e	1 ± 5.8 (5 μM)	1 ± 5.8
11	30 ± 1.5* (10 μM)	23 ± 5.8*
34	27 ± 6.8* (5 μM)	27 ± 6.8*
37	34 ± 0.2* (5 μM)	34 ± 0.2*

[a] Rat primary cortical cells (E18) were pretreated with different concentrations of test compounds for 24 h and subsequently exposed to glutamate (0.5 μM) for 24 h. Test compounds were present in the culture medium during glutamate exposure and were dissolved in DMSO. Glutamate was dissolved in PBS (pH 7.4). The final concentration of DMSO was < 5 μL/mL medium. Statistical analysis of data was performed using analysis of variance followed by Dunnett's test. The significance of results was set at $p < 0.05$. Values are expressed as % mean ± standard error of the mean. Asterisk (*) labeled values denote significant difference at $p < 0.05$ when compared with control glutamate group. Values in parentheses represent the best concentrations of their respective compounds.

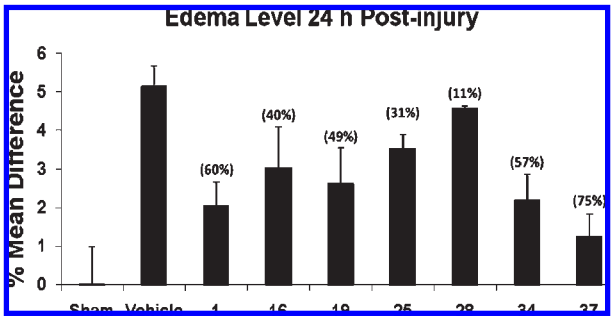


Figure 3. Effect of compound on edema level 24 h post-TBI. Values are expressed as mean ± SEM. Test compounds (8 mg/kg) were administered twice; first dose IP injections after 1 h post-TBI and second dose SC at 6 h post-TBI. At 24 h, animals were sacrificed and brain sections were removed for edema assay. Values in parentheses represent percent decrease in edema level compared to vehicle. The percent water content of each sample was calculated as [(wet wt − dry wt)/wet weight] × 100. The percent difference in water content of the area surrounding the injury was compared to an area distal to the injury [(contusion area − distal area/distal area) × 100].

of allopregnanolone, showed the greatest edema reduction among the allopregnanolone isomer group. Perhaps most notable was the activity of oxime derivative **37**, which showed a better average reduction in edema level compared to progesterone.

Conclusions

Several novel analogues of progesterone (**1**) and its natural metabolite allopregnanolone (**2**) were synthesized and screened for solubility as well as for their potential as neuroprotective agents. The use of an amino acid tether was shown to be an effective method for greatly enhancing the solubility of progesterone and other related steroidal compounds. Several compounds have shown nearly equivalent activity to progesterone and allopregnanolone in an in vitro assay designed to assess their ability to enhance neuronal cell survival. The 3β-hydroxy derivative **6b** and the oxime derived compound **37** both showed equivalent capacity relative to progesterone for reducing cerebral edema following cranial injury in a whole animal model of traumatic brain injury. Future work will seek to examine the pharmacokinetic properties of these compounds as well as to further evaluate their effectiveness through behavioral observation studies.

Experimental Section

General. Reaction progress was monitored via thin-layer chromatography (TLC) on precoated glass-backed plates (silica gel 60 Å F_{254}, 0.25 mm thickness). Flash chromatography was carried out with silica gel 60 Å (230−400 mesh) and automated chromatography was performed on an Isco Combiflash Companion. Unless otherwise stated, organic extracts were dried over commercially available magnesium sulfate and the solvents were removed by rotary evaporation. Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on either a 400 or 600 MHz Varian Inova spectrometer in deuterated chloroform ($CDCl_3$) and referenced to the residual solvent peak (^{1}H δ 7.27 ppm, ^{13}C δ 77.23 ppm) unless otherwise noted. Mass spectra were obtained on either a VG 70-S Nier Johnson or JEOL mass spectrometer. All prepared compounds were determined to be ≥95% pure, as assessed by combustion analysis. Combustion analyses were performed by Atlantic Microlab (Norcross, GA).

3β-Hydroxy-pregn-4-en-20-one (3). Progesterone (3.14 g, 10.0 mmol) was added with cerium chloride heptahydrate (3.73 g, 10.0 mmol, 1.00 equiv) to a three-necked 250 mL round-bottomed flask (RBF) with thermometer. Methanol (100 mL) was added under argon, and the solution was chilled to −20 °C. Sodium borohydride (0.189 g, 5.00 mmol, 0.500 equiv) was then added in bulk. After 10 min, 37 mL of acetone was added and the solution was warmed to ambient temperature. Water (25 mL) was added, and the solvent volume was reduced by approximately 100 mL. The aqueous layer was extracted with ether. The organic layers were combined, washed with brine, dried, filtered, and concentrated to give 3.14 g white solid. The solid was prepared as a silica cake and eluted from a 500 mL silica column with 20−25% EtOAc in hexanes. Initially eluting pure fractions were combined and concentrated to give 1.56 g of white solid that was 90% pure as determined by proton NMR (other 10% was progesterone). White solid (44% yield); R_f = 0.38 (1:1 EtOAc/hexanes, PMA stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.29 (d, 1 H, J = 1.6 Hz), 4.18−4.12 (m, 1 H), 2.51 (t, 1 H, J = 8.8 Hz), 2.25−0.77 (m, 20 H), 2.11 (s, 3 H), 1.04 (s, 3 H), 0.62 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.9, 147.4, 123.8, 68.1, 63.9, 56.5, 54.5, 44.3, 39.1, 37.5, 36.1, 35.6, 33.1, 32.3, 31.7, 29.6, 24.6, 22.9, 21.2, 19.1, 13.6. IR (solid): 3495, 2927, 2847, 1691, 1362, 1038 cm^{-1}. HRMS-ESI m/z 299.2379 ($[M + H - H_2O]^+$, $C_{21}H_{31}O$ requires 299.2369).

3β-Hydroxy-pregn-4-en-20-one, *N*-Fmoc-L-valine Vster (4b). An oven-dried 50 mL RBF was charged with compound **3** (0.352 g, 1.00 mmol), *N*-Fmoc-L-valine (0.373 g, 1.10 mmol, 1.10 equiv), and 4-dimethylaminopyridine (0.0122 g, 0.100 mmol, 0.100 equiv). The flask was sealed, evacuated, and inert gas flushed and 15 mL of anhydrous dichloromethane was added, followed by addition of 1.10 mL (1.10 mmol, 1.10 equiv) of 1 M dicyclohexylcarbodiimide in dichloromethane. The solution was stirred overnight and then filtered through celite. The filtrate was concentrated, prepared as a silica cake, and eluted on a 40 g silica column with a 0−25% EtOAc in hexanes gradient. The main product was isolated as 0.554 g (87%) clear oil that foamed on drying. R_f = 0.40 (1:1 EtOAc/hexanes, PMA stain). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.78 (d, 2H, J = 7.2 Hz), 7.63−7.61 (m, 2H), 7.41 (t, 2H, J = 7.2 Hz), 7.33 (t, 2H, J = 7.2 Hz), 5.35 (d, 1H, J = 9.0 Hz), 5.31 (t, 1H, J = 7.8 Hz), 5.21 (s, 1H), 4.40 (d, 2H, J = 7.2 Hz), 4.31 (dd, 1H, J = 9.0, 4.2 Hz), 4.25 (t, 1H, J = 7.2 Hz), 2.52 (t, 1H, J = 9.0 Hz), 2.23−2.16 (m, 3H), 2.12 (s, 3H), 2.05−1.96 (m, 3H), 1.78−1.55 (m, 6H), 1.50−1.33 (m, 4H), 1.25−1.10 (m, 2H), 1.06 (s, 3H), 1.00 (d, 3H, J = 7.2 Hz), 0.93 (d, 3H, J = 7.2 Hz), 0.90−0.79 (m, 2H), 0.64 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 209.8, 172.1, 156.5, 149.8, 144.2, 144.0, 141.5, 127.9, 127.2, 125.3, 120.2, 118.9, 118.8, 72.2, 72.1, 67.2, 63.8, 59.3, 59.2, 56.5, 54.2, 47.5, 47.4, 44.3, 39.0, 37.5, 36.0, 35.0, 33.0, 32.3, 31.6, 25.2, 24.6, 23.0, 19.2, 19.1, 19.0, 17.7, 13.6. IR (solid): 3360, 2936, 1701, 1390, 1352, 1198, 740 cm^{-1}.

3β-Hydroxy-pregn-4-en-20-one, L-Valine Ester (5b). A 25 mL RBF was charged with compound **4b** (0.340 g, 0.533 mmol). The flask was evacuated and inert gas flushed and 5 mL of acetonitrile was added. Piperidine (0.527 mL, 5.33 mmol, 10.0 equiv) was added, and the solution was stirred at room temperature for 30 min. The solvent was removed with addition of toluene for complete removal of piperidine. A white solid formed that was redissolved in a minimum amount of toluene, loaded onto a 12 g silica column, and eluted with 0−75% EtOAc in hexanes over 35 min. Main product containing fractions were combined and dried to give 0.196 g (89%) white foam. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.29−5.23 (m, 1H), 5.20 (d, 1H, J = 1.6 Hz), 3.27 (d, 1H, J = 4.8 Hz), 2.52 (t, 1H, J = 9.2 Hz), 2.36−1.93 (m, 6H), 2.11 (s, 3H), 1.79−1.08 (m, 14H), 1.06 (s, 3H), 0.98 (d, 3H, J = 6.8 Hz), 0.95−0.77 (m, 3H), 0.90 (d, 3H, J = 6.8 Hz), 0.62 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.8, 175.6, 149.3, 119.3, 71.3, 63.8, 60.2, 56.4, 54.2, 44.3, 39.0, 37.5, 36.0, 35.2, 33.0, 32.3 (2 C), 31.7, 25.3, 24.6, 22.9, 21.1, 19.6, 19.0, 17.3, 13.6. IR (solid): 2934, 2843, 1724, 1705, 1384, 1354, 1166, 1146, 978, 873, 852 cm. HRMS-ESI m/z 416.3156 ($[M + H]^+$, $C_{26}H_{42}NO_3$ requires 416.3159).

3β-Hydroxy-pregn-4-en-20-one, L-Valine Ester Hydrochloride (6b). A 10 mL RBF was charged with compound **5b** (0.083 g, 0.20 mmol) and the flask was evacuated and flushed with argon. Anhydrous ether (2 mL) was added, and the solution was chilled in an ice bath. Hydrogen chloride solution (0.10 mL 2.0 M in ether, 0.20 mmol, 1.0 equiv) was added dropwise. A white precipitate formed in solution. The solution was stirred for 30 min. The precipitate was filtered and washed with chilled ether. The product was isolated as 68 mg (75%) white solid; mp 159−161 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.80 (bs, 2H), 5.35 (t, 1H, J = 7.8 Hz), 5.27 (s, 1H), 3.89 (d, 1H, J = 4.8 Hz), 2.53−2.46 (m, 2H), 2.23−0.78 (m, 21H), 2.12 (s, 3H), 1.17 (d, 3H, J = 7.2 Hz), 1.14 (d, 3H, J = 7.2 Hz), 1.06 (s, 3H), 0.64 (s, 3H). ^{13}C NMR (150 MHz, 60 °C, $CDCl_3$) δ 209.1, 168.5, 150.3, 118.6, 73.6, 64.0, 58.9, 56.7, 54.3, 44.3, 39.3, 37.7, 36.3, 35.0, 33.3, 32.5, 31.5, 30.3, 25.3, 24.7, 23.3, 21.4, 19.3, 18.7, 18.6, 13.6. IR (film): 2932, 2848, 2600, 1737, 1702, 1380, 1355, 1219, 1109 cm^{-1}. HRMS-ESI m/z 416.3160 ($[M - Cl]^+$, $C_{26}H_{42}NO_3$ requires 416.3159). Anal. ($C_{26}H_{42}ClNO_3$ + $^1/_2H_2O$) C, H, N.

Compounds **6a**−**6e** were prepared according to the methods as described for compound **6b**:

3β-Hydroxy-pregn-4-en-20-one, L-alanine Ester Hydrochloride (6a). White solid (52% yield); mp 112−114 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (bs, 2H), 5.25 (t, 1H, J = 7.6 Hz), 5.14 (s, 1H), 4.12 (d, 1H, J = 7.6 Hz), 2.64 (bs, 1H), 2.43 (t, 1H, J = 8.8 Hz), 2.20−0.68 (m, 21H), 2.04 (s, 3H), 1.65 (d, 3H, J = 7.2 Hz), 0.99 (s, 3H), 0.57 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.7, 170.1, 150.1, 118.4, 73.4, 63.8, 56.4, 54.1, 49.5, 44.2, 39.0, 37.5, 36.0, 35.0, 33.0, 32.3, 31.7, 25.0, 24.6, 23.0, 21.2, 19.0, 16.4, 13.5. IR (film): 2934, 2849, 1741, 1703, 1237, 1207, 1113, 916, 731 cm^{-1}. HRMS-ESI m/z 388.2847 ([M − Cl]$^+$, $C_{24}H_{38}NO_3$ requires 388.2846). Anal. ($C_{24}H_{38}ClNO_3 + {}^1/_2H_2O$) C, H, N.

3β-Hydroxy-pregn-4-en-20-one, L-Phenylalanine Ester Hydrochloride (6c). White solid (51% yield); mp 148−150 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.71 (bs, 2H), 7.30−7.21 (m, 5H), 5.17 (bs, 1H), 4.97 (s, 1H), 4.32 (bs, 1H), 3.47−3.29 (m, 2H), 2.48 (t, 1H, J = 8.6 Hz), 2.20−0.68 (m, 20H), 2.10 (s, 3H), 0.97 (s, 3H), 0.60 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.7, 168.8, 149.9, 134.2, 130.1, 129.0, 127.8, 118.2, 73.6, 63.8, 56.4, 54.5, 54.1, 44.2, 39.0, 37.4, 36.6, 36.0, 34.8, 33.0, 32.2, 31.7, 24.9, 24.6, 23.0, 21.2, 18.9, 13.6. IR (film): 2929, 2848, 1732, 1701, 1233, 1202, 1109, 912, 729, 700 cm^{-1}. HRMS-ESI m/z 464.3160 ([M − Cl]$^+$, $C_{30}H_{42}NO_3$ requires 464.3159). Anal. ($C_{30}H_{42}ClNO_3 + {}^1/_2H_2O$) C, H, N.

3β-Hydroxy-pregn-4-en-20-one, L-Proline Ester Hydrochloride (6d). White solid (38% yield); mp 130−131. ^{1}H NMR (400 MHz, DMSO) δ 10.32 (bs, 1H), 9.02 (bs, 1H), 5.26 (s, 1H), 5.22 (s, 1H), 4.33 (s, 1H), 3.40 (s, 1H), 3.20 (d, 2H, J = 7.2 Hz), 2.56 (t, 1H, J = 8.6 Hz), 2.30−0.73 (m, 23H), 2.05 (s, 3H), 1.02 (s, 3H), 0.54 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.6, 168.9, 150.6, 118.0, 74.1, 63.8, 59.5, 56.4, 54.0, 46.6, 44.2, 38.9, 37.5, 35.9, 34.8, 33.0, 32.6, 31.7, 29.4, 25.1, 24.5, 24.0, 22.9, 21.1, 19.0, 13.5. IR (film): cm^{-1}. HRMS-ESI m/z 414.3005 ([M − Cl]$^+$, $C_{26}H_{40}NO_3$ requires 414.3003). Anal. ($C_{26}H_{40}ClNO_3 + {}^1/_2H_2O$) C, H, N.

3β-Hydroxy-pregn-4-en-20-one, L-Tryptophan Ester Hydrochloride (6e). White solid (43% yield); mp 118−120 °C. ^{1}H NMR (400 MHz, DMSO) δ 11.1 (bs, 1H), 8.62 (bs, 2H), 7.53 (d, 1H, J = 7.6 Hz), 7.36 (d, 1H, J = 7.6 Hz), 7.24 (s, 1H), 7.08 (t, 1H, J = 7.4 Hz), 6.99 (t, 1H, J = 7.4 Hz), 5.07 (bs, 1H), 4.73 (s, 1H), 4.14 (bs, 1H), 3.46−3.20 (m, 2H), 2.54 (t, 1H, J = 8.8 Hz), 2.20−0.67 (m, 20H), 2.05 (s, 3H), 0.95 (s, 3H), 0.53 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.7, 169.3, 150.3, 136.4, 126.9, 126.6, 122.0, 119.3, 118.5, 118.1, 112.1, 105.9, 74.1, 63.8, 56.4, 54.0, 53.6, 44.2, 39.0, 37.5, 35.9, 34.7, 33.0, 32.3, 31.7, 26.1, 25.0, 24.6, 23.0, 21.1, 19.0, 13.6. IR (film): 2929, 2849, 1732, 1701, 1456, 1435, 1354, 1218, 1108, 730 cm^{-1}. HRMS-ESI m/z 503.3271 ([M − Cl]$^+$, $C_{32}H_{43}N_2O_3$ requires 503.3268). Anal. ($C_{32}H_{43}ClN_2O_3 + {}^3/_4H_2O$) C, H, N.

Pregn-4-ene-3,20-diol (20*S*) (7). An oven-dried RBF was charged with 25 mL of anhydrous THF and chilled in an ice bath. Lithium aluminum hydride (4.50 mL 2.0 M in THF, 9.00 mmol, 2.25 equiv) was added. A separate ∼10 mL solution of progesterone (1.26 g, 4.00 mmol) in anhydrous THF was prepared in a dry flask. The solution was transferred to the reaction flask dropwise over 30 min. The mixture was heated under reflux for 1 h, cooled to room temperature, and quenched by the addition of EtOAc, followed by aqueous sodium sulfate. Solid sodium sulfate was added to remove excess water. The remaining salts were filtered and washed with THF. The organic filtrates were combined and concentrated to give 1.24 g (90%) white crystalline solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.27 (d, 1H, J = 0.8 Hz), 4.19−4.10 (m, 1H), 3.76−3.68 (m, 1H), 2.24−0.71 (m, 22H), 1.13 (d, 3H, J = 6.0 Hz), 1.05 (s, 3H), 0.77 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 147.7, 123.6, 70.7, 68.1, 58.6, 55.8, 54.6, 42.6, 40.1, 37.5, 35.9, 35.6, 33.3, 32.4, 29.7, 25.8, 24.6, 23.8, 21.1, 19.1, 12.7. IR (solid): 3300, 2920, 2865, 1435, 1375, 1021, 853 cm^{-1}. HRMS-ESI m/z 301.2524 ([M + H − H_2O]$^+$, $C_{21}H_{33}O$ requires 301.2526).

20(*S*)-Hydroxypregn-4-en-3-one (8). A 100 mL RBF was charged with crude compound **7** (1.00 g) and manganese dioxide (5.00 g, activated by heating in oven for 2 days then cooled in a desiccator) and the reactants were suspended in 30 mL of chloroform. The mixture was stirred at room temperature overnight. The mixture was then filtered through a pad of celite and rinsed with chloroform. The clear, colorless filtrate was evaporated to dryness to give an off-white solid. The solid was recrystallized from EtOAc/hexanes to give 0.565 g (57%) white solid; R_f = 0.26 (1:1 EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.73 (s, 1H), 3.77−3.70 (m, 1H), 2−47−0.91 (m, 21H), 1.19 (s, 3H), 1.15 (d, 3H, J = 6.0 Hz), 0.80 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.9, 171.8, 124.0, 70.7, 58.5, 55.5, 54.0, 42.5, 39.8, 38.8, 35.9, 35.6, 34.2, 33.1, 32.2, 25.8, 24.6, 24.0, 21.1, 17.6, 12.6. IR (solid): 3525, 2939, 2864, 1670, 1609, 1117, 857 cm^{-1}. HRMS-ESI m/z 317.2473 ([M + H]$^+$, $C_{21}H_{32}O_2$ requires 317.2475).

20(*S*)-Hydroxypregn-4-en-3-one, *N*-Fmoc-L-valine Ester (9). Prepared according to the method described for compound **4b**. White foam (87% yield); R_f = 0.65 (1:1 EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, 2H, J = 8.0 Hz), 7.62 (d, 2H, J = 7.6 Hz), 7.42−7.37 (m, 2H), 7.33 (t, 2H, J = 7.2 Hz), 5.71 (s, 1H), 5.41 (d, 1H, J = 8.8 Hz), 4.97−4.90 (m, 1H), 4.48−4.16 (m, 4H), 2.43−0.80 (m, 21H), 1.20 (d, 3H, J = 6.0 Hz), 1.08 (s, 3H), 0.99 (d, 3H, J = 6.8 Hz), 0.94 (d, 3H, J = 7.2 Hz), 0.67 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 200.0, 171.8, 171.7, 156.4, 144.2, 143.9, 141.5, 127.9, 127.3, 125.3, 124.0, 120.2, 74.4, 67.3, 59.2, 55.4, 55.0, 53.9, 47.4, 42.4, 39.1, 38.7, 35.7, 35.6, 34.1, 33.0, 32.1, 31.5, 25.6, 24.4, 21.1, 20.0, 19.1, 17.8, 17.4, 12.7. IR (solid): 3307, 2935, 1716, 1668, 1229, 1029, 739 cm^{-1}. HRMS-ESI m/z 638.3847 ([M + H]$^+$, $C_{41}H_{52}NO_5$ requires 638.3840).

20(*S*)-Hydroxypregn-4-en-3-one, L-Valine Ester (10). Prepared according to the method described for compound **5b**. White powdery solid (40% yield); R_f = 0.06 (1:1 EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.72 (s, 1H), 4.93−4.86 (m, 1H), 3.23 (d, 1H, J = 4.4 Hz), 2.46−2.23 (m, 5H), 2.10−0.80 (m, 18H), 1.17 (s, 3H), 1.16 (d, 3H, J = 6.4 Hz), 0.98 (d, 3H, J = 7.2 Hz), 0.88 (d, 3H, J = 6.8 Hz), 0.68 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.8, 175.1, 171.5, 124.0, 73.4, 59.9, 55.4, 55.1, 54.0, 42.5, 39.2, 38.8, 35.9, 35.6, 34.2, 33.0, 32.2 (2 C), 25.6, 24.4, 21.1, 20.0, 19.5, 17.6, 17.1, 12.7. IR (film): 2933, 1721, 1672, 1381, 1187, 1071, 864 cm^{-1}. HRMS-ESI m/z 416.3156 ([M + H]$^+$, $C_{26}H_{42}NO_3$ requires 416.3159).

20(*S*)-Hydroxypregn-4-en-3-one, L-Valine Ester Hydrochloride (11). Prepared according to the method described for compound **6b**. Off-white solid (59% yield); mp 133−135 °C. ^{1}H NMR (400 MHz, DMSO) δ 8.51 (bs 2H), 5.63 (s, 1H), 4.86 (q, 1H, J = 10.0, 5.6 Hz), 3.79 (bs 1H), 2.46−0.82 (m, 23H), 1.15 (d, 3H, J = 6.0 Hz), 1.14 (s, 3H), 0.99 (d, 3H, J = 6.4 Hz), 0.95 (d, 3H, J = 6.4 Hz), 0.66 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.8, 171.5, 168.2, 124.0, 76.0, 58.6, 55.5, 54.8, 54.0, 42.5, 39.2, 38.8, 35.9, 35.6, 34.2, 33.0, 32.2, 30.1, 25.5, 24.3, 21.1, 19.9, 18.6, 18.5, 17.6, 12.7. IR (solid): 2935, 2870, 1733, 1667, 1378, 1331, 1228, 1071, 863 cm^{-1}. HRMS-ESI m/z 416.3152 ([M − Cl]$^+$, $C_{26}H_{42}NO_3$ requires 416.3159). Anal. ($C_{26}H_{42}ClNO_3 + H_2O$) C, H, N.

3β-Hydroxy-5α-pregnan-20-one (13). An oven-dried 500 mL RBF was charged with 10% palladium on carbon (0.400 g) and compound **12** (4.00 g, 12.6 mmol) and the flask was evacuated and flushed with argon. Absolute ethanol (200 mL) was added, and the flask was flushed with hydrogen. The reaction was stirred at room temperature for 4 h. The mixture was filtered through celite, and the recovered filtrate was concentrated to reveal a white solid of mass 4.08 g. The solid was recrystallized from hexanes/EtOAc (∼3:1 total 175 mL) to give 3.19 g of white solid. A second recrystallization provided an additional 0.43 g for a total of 3.62 g (90%) white crystalline solid; R_f = 0.38 (1:1 EtOAc/hexanes, PMA). ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.62−3.57 (m, 1H), 2.52 (t, 1H, J = 9.0 Hz), 2.18−1.97 (m, 2H), 2.11 (s, 3H), 1.83−0.86 (m, 20H), 0.81 (s, 3H), 0.71−0.65

(m, 1H), 0.60 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 210.0, 71.5, 64.0, 56.9, 54.4, 45.0, 44.5, 39.3, 38.3, 37.2, 35.7 (2C), 32.2, 31.7 (2C), 28.8, 24.6, 23.0, 21.5, 13.7, 12.5. IR (solid): 3426, 3375, 2930, 2845, 1697, 1682, 1385, 1036 cm^{-1}. HRMS-ESI m/z 319.2629 ([M + H]$^+$, $C_{21}H_{35}O_2$ requires 319.2632).

3β-Hydroxy-5α-pregnan-20-one, *N*-Fmoc-L-valine Ester (14). Prepared according to the method described for compound **4b**. White foam (90% yield); R_f = 0.69 (1:1 EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, 2H, J = 7.6 Hz), 7.61 (d, 2H, J = 6.8 Hz), 7.41 (t, 2H, J = 7.6 Hz), 7.33 (t, 2H, J = 7.2 Hz), 5.34 (d, 1H, J = 9.2 Hz), 4.83−4.73 (m, 1H), 4.49−4.20 (m, 4H), 2.52 (m, 1H), 2.23−0.66 (m, 23H), 2.12 (s, 3H), 0.98 (d, 3H, J = 6.8 Hz), 0.92 (d, 3H, J = 6.8 Hz), 0.83 (s, 3H), 0.61 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.9, 171.8, 156.4, 144.1/144.0, 141.5, 127.9, 127.3, 125.3, 120.2, 75.0, 67.2, 64.0, 59.2, 56.7, 54.1, 47.4, 44.8, 44.4, 39.2, 36.9, 35.7, 35.6, 34.1, 32.1, 31.8, 31.6, 28.6, 27.6, 24.6, 23.0, 21.4, 19.1, 17.7, 13.7, 12.4. IR (solid): 3344, 2931, 1701, 1449, 1200, 739 cm^{-1}. HRMS-ESI m/z 640.3979 ([M + H]$^+$, $C_{41}H_{54}NO_5$ requires 640.3983).

3β-Hydroxy-5α-pregnan-20-one, L-Valine Ester (15). Prepared according to the method described for compound **5b**. Clear/white semisolid (99% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.77−4.65 (m, 1H), 3.26 (d, 1H, J = 4.4 Hz), 2.87 (bs, 2H), 2.51 (t, 1H, J = 9.2 Hz), 2.36−0.62 (m, 23H), 2.09 (s, 3H), 0.96 (d, 3H, J = 7.2 Hz), 0.88 (d, 3H, J = 6.4 Hz), 0.81 (s, 3H), 0.58 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 210.0, 174.7, 74.4, 64.0, 59.7, 56.8, 54.2, 44.8, 44.4, 39.2, 36.9, 35.7, 35.6, 34.2, 32.2, 32.1, 31.8, 28.6, 27.6, 24.6, 23.0, 21.4, 19.3, 17.4, 13.7, 12.4. IR (solid): 2927, 2849, 1729, 1703, 1385, 1358, 1224, 1006 cm^{-1}. HRMS-ESI m/z 418.3310 ([M + H]$^+$, $C_{26}H_{44}NO_3$ requires 418.3316).

3β-Hydroxy-5α-pregnan-20-one, L-Valine Ester Hydrochloride (16). Compound **15** (0.317 g, 0.759 mmol) was dissolved in ~2:1 anhydrous ether/CH_2Cl_2 (6 mL total) under argon. The solution was chilled in an ice bath and 0.759 mL (0.759 mmol, 1.0 equiv) 1 M HCl in ether solution was added slowly dropwise. A white precipitate was observed in solution. The solution was stirred at 0 °C for 30 min and then filtered. The precipitate was washed with ice chilled ether. The product was recovered as 0.175 g (51%) slightly off-white solid; mp 185−187 °C. ^{1}H NMR (400 MHz, DMSO) δ 8.55 (bs, 1H), 4.80−4.69 (m, 1H), 3.80 (s, 1H), 3.37 (s, 1H), 2.56 (t, 1H, J = 8.8 Hz), 2.22−2.12 (m, 1H), 2.08−0.65 (m, 23H), 2.05 (s, 3H), 0.98 (d, 3H, J = 7.2 Hz), 0.93 (d, 3H, J = 6.8 Hz), 0.79 (s, 3H), 0.50 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.9, 167.9, 76.3, 63.9, 58.6, 56.7, 54.2, 44.8, 44.4, 39.1, 36.9, 35.6, 35.5, 33.9, 32.1, 31.8, 30.1, 28.6, 27.4, 24.6, 22.9, 21.4, 18.5, 18.4, 13.6, 12.4. IR (solid): 3369, 2927, 2850, 2620, 1732, 1703, 1382, 1359, 1222, 1002 cm^{-1}. HRMS-ESI m/z 418.3316 ([M − Cl]$^+$, $C_{26}H_{44}NO_3$ requires 418.3316). Anal. ($C_{26}H_{44}ClNO_3$) C, H.

Allopregnanolone (2). An oven-dried 100 mL RBF with magnetic stir bar was charged with compound **13** (1.59 g, 5.00 mmol) and 15 mL of anhydrous THF. Diethylazodicarboxylate (2.85 mL 40% soln in toluene, 6.25 mmol, 1.25 equiv) was added, followed by trifluoroacetic acid (0.482 mL, 6.25 mmol, 1.25 equiv), and the flask was set in a room temperature water bath. To this pale-amber suspension was added triphenylphosphine (1.64 g, 6.25 mmol, 1.25 equiv). Sodium benzoate (0.901 g, 6.25 mmol, 1.25 equiv) was then added and the suspension was stirred under argon for 24 h at room temperature. The THF was completely removed with methanol addition/evaporation. Methanol (20 mL) was then added. The flask was fitted with a drying tube topped condenser and set for reflux. After 24 h, the methanol was removed and the remaining solid was redissolved in CH_2Cl_2. The organic layer was washed with water (3 × 20 mL). The aqueous layers were combined and extracted with CH_2Cl_2. The organic layers were combined, dried, filtered, and concentrated to give a white solid. The solid was prepared as a silica cake and eluted with 0−35% EtOAc in hexanes on a 120 g silica column over 40 min. Main product containing fractions were combined and concentrated to give 1.46 g (92%) white solid. R_f = 0.38 (1:1 EtOAc/hexanes, PMA). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.06−4.04 (m, 1H), 2.53 (t, 1H, J = 9.2 Hz), 2.19−1.96 (m, 2H), 2.11 (s, 3H), 1.73−0.74 (m, 21H), 0.78 (s, 3H), 0.60 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.1, 66.7, 64.0, 56.9, 54.4, 44.5, 39.3 (2C), 36.3, 36.0, 35.7, 32.4, 32.1, 31.8, 29.2, 28.6, 24.6, 22.9, 21.0, 13.7, 11.4. IR (solid): 3252, 2913, 2850, 1706, 1446, 1351, 1004 cm^{-1}. HRMS-ESI m/z 319.2633 ([M + H]$^+$, $C_{21}H_{35}O_2$ requires 319.2632).

3α-Hydroxy-5α-pregnan-20-one, *N*-Fmoc-L-valine Ester (17). Prepared according to the method described for compound **4b**. White foam (87% yield); R_f = 0.65 (1:1 EtOAc/hexanes, PMA stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, 2H, J = 7.6 Hz), 7.64 (d, 2H, J = 7.6 Hz), 7.42 (t, 2H, J = 7.2 Hz), 7.33 (t, 2H, J = 7.2 Hz), 5.37 (d, 1H, J = 8.4 Hz), 5.13 (s, 1H), 4.46−4.33 (m, 3H), 4.27 (t, 1H, J = 7.2 Hz), 2.49−0.68 (m, 24H), 2.09 (s, 3H), 1.02 (d, 3H, J = 7.2 Hz), 0.95 (d, 3H, J = 7.2 Hz), 0.79 (s, 3H), 0.58 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 210.0, 171.7, 156.5, 144.2/143.9, 141.5, 127.9, 127.3, 125.4, 120.2, 71.7, 67.3, 63.9, 59.3, 56.7, 54.3, 47.5, 44.4, 40.2, 39.1, 35.9, 35.5, 33.2, 33.0, 31.8 (2C), 31.7, 28.3, 26.3, 24.5, 22.9, 21.0, 19.3, 17.7, 13.6, 11.5. IR (film): 3364, 2931, 1698, 1387, 1353, 1232, 1036, 739 cm^{-1}. HRMS-ESI m/z 640.3989 ([M + H]$^+$, $C_{41}H_{54}NO_5$ requires 640.3988).

3α-Hydroxy-5α-pregnan-20-one, L-Valine Ester (18). A 25 mL RBF was charged with compound **17** (0.320 g, 0.500 mmol), 5 mL ACN, and 3 mL of DMF. Piperidine (0.494 mL, 5.00 mmol, 10.0 equiv) was added. The solution was stirred at room temperature for 30 min. Toluene was added and the solution was concentrated 3 times with addition of toluene. The pale-amber oil was loaded in a minimum amount of toluene onto a 12 g silica column. The column was eluted with 0−100% EtOAc in hexanes over 40 min. Main product fractions were combined to give 0.196 g of (94%) sticky white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.09 (t, 1H, J = 2.4 Hz), 3.89 (bs, 2H), 3.37 (d, 1H, J = 4.8 Hz), 2.52 (t, 1H, J = 9.0 Hz), 2.19−0.76 (m, 23H), 2.11 (s, 3H), 1.01 (d, 3H, J = 6.6 Hz), 0.93 (d, 3H, J = 6.6 Hz), 0.80 (s, 3H), 0.61 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 210.0, 174.6, 70.9, 64.0, 59.7, 56.9, 54.3, 44.4, 40.3, 39.2, 36.0, 35.6, 33.2, 33.0, 32.2, 32.0, 31.7, 28.4, 26.4, 24.5, 23.0, 21.0, 19.4, 17.3, 13.7, 11.5. IR (solid): 2931, 2856, 1724, 1701, 1388, 1356, 1227, 1153, 975 cm^{-1}. HRMS-ESI m/z 418.3306 ([M + H]$^+$, $C_{26}H_{44}NO_3$ requires 418.3302).

3α-Hydroxy-5α-pregnan-20-one, L-Valine Ester Hydrochloride (19). Prepared according to the method described for compound **6b**. Slightly off-white solid (55% yield); mp 123−124 °C. ^{1}H NMR (600 MHz, DMSO) δ 8.56 (bs, 1H), 5.06 (s, 1H), 3.84 (s, 1H), 2.59−0.66 (m, 26H), 2.05 (s, 3H), 1.02 (d, 3H, J = 7.2 Hz), 0.96 (d, 3H, J = 6.6 Hz), 0.77 (s, 3H), 0.51 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 209.9, 168.3, 73.2, 64.0, 58.7, 56.8, 54.2, 44.4, 40.2, 39.1, 35.9, 35.6, 33.2, 32.8, 31.9, 31.7, 30.2, 28.3, 26.2, 24.5, 23.0, 21.0, 18.6, 18.4, 13.6, 11.6. IR (film): 2927, 2852, 2620, 1733, 1702, 1382, 1353, 1226, 1155, 974 cm^{-1}. HRMS-ESI m/z 418.3314 ([M − Cl]$^+$, $C_{26}H_{44}NO_3$ requires 418.3316). Anal. ($C_{26}H_{44}ClNO_3 + {}^1/_4H_2O$) C, H.

5β-Pregnan-3,20-dione (20). A three-necked 500 mL RBF was charged with progesterone (2.00 g, 6.36 mmol), 5% Pd/$CaCO_3$ (0.180 g, 9% w/w), 200 mL absolute ethanol, and KOH (0.360 g in 1 mL DI). The flask was evacuated and flushed with hydrogen and the reaction stirred for 1 h. The ethanol was removed and the residue was redissolved in ether and washed with water. The water layer was extracted with ether (2 × 50 mL). The aqueous layer was then acidified to pH < 3 with 1 M HCl and extracted with ether. The organic layers were combined, dried, filtered, and concentrated to give an off-white solid of mass 2.08 g. The sample was loaded in a minimum amount of toluene onto a 120 g silica column and eluted with 0−35% ea in hex gradient. The main product was recovered as 1.20 g (60%) white solid; R_f = 0.51 (1:1 EtOAc/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.69 (t, 1H, J = 15 Hz), 2.55 (t, 1H, J = 9.0 Hz), 2.34 (dt, 1H, J = 14.4, 5.4 Hz), 2.21−1.09 (m, 20H), 2.12 (s, 3H), 1.02 (s, 3H), 0.64

(s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 213.3, 209.7, 64.0, 56.8, 44.5, 44.4, 42.5, 40.9, 39.3, 37.4, 37.1, 35.7, 35.1, 31.8, 26.7, 25.9, 24.6, 23.1, 22.8, 21.4, 13.6. IR (film): 2931, 2852, 1716, 1698, 1439, 1352 cm^{-1}. HRMS-ESI m/z 317.2473 ([M + H]$^+$, $C_{21}H_{33}O_2$ requires 317.2475).

3-Hydroxy-5β-pregnan-20-one (21/22). A 250 mL RBF was charged with compound **20** (1.00 g, 3.16 mmol) and 40 mL absolute ethanol. The solution was warmed in an oil bath to 50 °C and sodium borohydride (0.179 g, 4.74 mmol, 1.50 equiv) was added. The reaction was stirred for 10 min and 75−100 mL of hot water was added until a slight cloudiness remained in solution. The solution was then allowed to cool gradually to room temperature and chilled in a 4 °C freezer for 3 h. The mixture was filtered, and the white solid was washed with 30% ethanol in DI. After drying, the recovered solids were loaded in a minimum amount of DCM onto a 120 g silica column and eluted with 0−25% EA/hex over 60 min. Main product containing fractions were combined and concentrated to give 0.710 g (71%) 3α-hydroxy-5β-pregnan-20-one (**21**) and 0.110 g (11%) 3β-hydroxy-5β-pregnan-20-one isomer (**22**). Major product (**21**): white solid (71% yield); R_f = 0.30 (1:1 EtOAc/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.67−3.62 (m, 1H), 2.53 (t, 1H, J = 9.6 Hz), 2.18−0.96 (m, 23H), 2.11 (s, 3H), 0.92 (s, 3H), 0.59 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 210.0, 71.9, 64.1, 56.9, 44.5, 42.2, 40.6, 39.4, 36.6, 36.0, 35.5, 34.8, 31.8, 30.7, 27.3, 26.6, 24.6, 23.5, 23.1, 21.0, 13.6. IR (film): 3391, 2927, 2847, 1702, 1352, 1040 cm^{-1}. HRMS-ESI m/z 319.2638 ([M + H]$^+$, $C_{21}H_{35}O_2$ requires 319.2632). Minor product (**22**): white solid (11% yield); R_f = 0.41 (1:1 EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.12 (t, 1H, J = 2.8 Hz), 2.53 (t, 1H, J = 9.2 Hz), 2.20−1.00 (m, 23H), 2.11 (s, 3H), 0.96 (s, 3H), 0.60 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 210.0, 67.2, 64.1, 57.0, 44.6, 39.9, 39.5, 36.7, 35.8, 35.4, 33.7, 31.8, 30.1, 28.0, 26.7, 26.4, 24.6, 24.1, 23.0, 21.3, 13.7. IR (film): 3330, 2924, 2871, 1701, 1352, 1032 cm^{-1}. HRMS-ESI m/z 319.2636 ([M + H]$^+$, $C_{21}H_{35}O_2$ requires 319.2632).

3α-Hydroxy-5β-pregnan-20-one, *N*-Fmoc-L-valine Ester (23). Prepared according to the method described for compound **4b**. White foam (81% yield); R_f = 0.66 (1:1 EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, 2H, J = 7.2 Hz), 7.63 (t, 2H, J = 6.4 Hz), 7.41 (dt, 2H, J = 7.6, 1.6 Hz), 7.33 (t, 2H, 7.6 Hz), 5.32 (d, 1H, J = 9.2 Hz), 4.87−4.78 (m, 1H), 4.48 (dd, 1H, J = 10.4, 6.4 Hz), 4.38−4.20 (m, 3H), 2.49−0.86 (m, 24H), 2.09 (s, 3H), 1.00 (d, 3H, J = 6.8 Hz), 0.94 (d, 2H, J = 8.0 Hz), 0.93 (s, 3H), 0.58 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 209.9, 171.7, 156.5, 144.2/143.8, 141.5, 127.9, 127.3, 125.4, 125.3, 120.2, 75.6, 67.2, 64.0, 59.3, 56.7, 47.4, 44.4, 42.0, 40.5, 39.3, 35.9, 35.1, 34.8, 32.3, 31.8, 31.5, 27.0, 26.9, 26.4, 24.6, 23.4, 23.0, 21.0, 19.2, 17.8, 13.6. IR (film): 3335, 2931, 2868, 1700, 1448, 1194, 1022, 740 cm^{-1}. HRMS-ESI m/z 640.3993 ([M + H]$^+$, $C_{41}H_{54}NO_5$ requires 640.3997).

3α-Hydroxy-5β-pregnan-20-one, L-Valine Ester (24). Prepared according to the method described for compound **5b**; white foam (97% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.84−4.72 (m, 1H), 3.26 (d, 1H, J = 4.8 Hz), 2.54 (t, 1H, J = 8.8 Hz), 2.18−0.85 (m, 25H), 2.11 (s, 3H), 0.99 (d, 3H, J = 7.2 Hz), 0.94 (s, 3H), 0.91 (d, 3H, J = 7.2 Hz), 0.60 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 209.9, 174.9, 74.9, 64.1, 60.0, 56.9, 44.5, 42.0, 40.6, 39.4, 36.0, 35.1, 34.8, 32.4, 32.2, 31.8, 27.1, 26.9, 26.5, 24.6, 23.5, 23.0, 21.0, 19.5, 17.3, 13.6. IR (film): 2929, 2867, 1726, 1703, 1384, 1357, 1174, 988 cm^{-1}. HRMS-ESI m/z 418.3310 ([M + H]$^+$, $C_{26}H_{44}NO_3$ requires 418.3316).

3α-Hydroxy-5β-pregnan-20-one, L-Valine Ester Hydrochloride (25). Prepared according to the method described for compound **6b**. White solid (71% yield); mp 152−154 °C. ^{1}H NMR (600 MHz, DMSO) δ 8.58 (bs, 1H), 4.79 (s, 1H), 3.79 (s, 1H), 2.58 (t, 1H, J = 9.0 Hz), 2.42−0.82 (m, 25H), 2.05 (s, 3H), 1.00 (d, 3H, J = 7.2 Hz), 0.95 (d, 3H, J = 6.6 Hz), 0.91 (s, 3H), 0.50 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 209.8, 168.0, 64.0, 58.7, 56.7, 44.5, 42.1, 40.6, 39.3, 36.0, 35.1, 34.8, 32.2, 31.7, 30.1, 27.0, 26.8, 26.4, 24.6, 23.4 (2C), 23.0, 21.0, 18.6, 18.5, 13.6. IR (solid): 2929, 2866, 2600, 1737, 1702, 1379, 1356, 1194, 979 cm^{-1}. HRMS-ESI m/z 418.3311 ([M − Cl]$^+$, $C_{26}H_{44}NO_3$ requires 418.3316). Anal. ($C_{26}H_{44}ClNO_3 + {}^1/_4H_2O$) C, H, N.

3β-Hydroxy-5β-pregnan-20-one, *N*-Fmoc-L-valine Ester (26). Prepared according to the method described for compound **4b**. White foam (73% yield); R_f = 0.66 (1:1 EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, 2H, J = 7.6 Hz), 7.62 (d, 2H, J = 7.2 Hz), 7.41 (t, 2H, J = 7.6 Hz), 7.32 (dt, 2H, J = 7.6, 1.2 Hz), 5.36 (d, 1H, J = 9.2 Hz), 5.19 (s, 1H), 4.50−4.28 (m, 3H), 4.24 (t, 1H, J = 7.2 Hz), 2.57−0.84 (m, 24H), 2.12 (s, 3H), 1.00 (d, 3H, J = 7.2 Hz), 0.98 (s, 3H), 0.93 (d, 3H, J = 6.4 Hz), 0.61 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.8, 171.7, 156.5, 144.1/144.0, 141.5, 127.9, 127.3, 125.3, 120.2, 72.3, 67.2, 64.0, 59.2, 56.9, 47.4, 44.5, 40.1, 39.4, 37.7, 35.8, 35.1, 31.8, 31.7, 31.0, 30.8, 26.6, 26.3, 25.2, 24.6, 24.1, 23.0, 21.3, 19.2, 17.7, 13.6. IR (solid): 3335, 2930, 2870, 1700, 1448, 1202, 1020, 739 cm^{-1}. HRMS-ESI m/z 640.3997 ([M + H]$^+$, $C_{41}H_{54}NO_5$ requires 640.3997).

3β-Hydroxy-5β-pregnan-20-one, L-Valine Ester (27). Prepared according to the method described for compound **5b**. White foam (93% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.15 (s, 1H), 3.34 (d, 1H, J = 4.8 Hz), 2.54 (t, 1H, J = 9.2 Hz), 2.20−0.86 (m, 25H), 2.11 (s, 3H), 1.00 (d, 3H, J = 7.2 Hz), 0.97 (s, 3H), 0.91 (d, 3H, J = 6.8 Hz), 0.60 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.9, 174.8, 71.4, 64.1, 60.1, 56.9, 44.6, 40.1, 39.4, 37.7, 35.8, 35.1, 32.3, 31.8, 31.0, 30.8, 26.6, 26.3, 25.3, 24.6, 24.1, 23.0, 21.3, 19.5, 17.3, 13.6. IR (film): 2929, 2868, 1723, 1703, 1447, 1385, 1357, 1152, 1019 cm^{-1}. HRMS-ESI m/z 418.3312 ([M + H]$^+$, $C_{26}H_{44}NO_3$ requires 418.3316).

3β-Hydroxy-5β-pregnan-20-one, L-Valine Ester Hydrochloride (28). Prepared according to the method described for compound **6b**. Slightly off-white solid (39% yield); mp 200−201 °C. ^{1}H NMR (600 MHz, DMSO) δ 8.47 (bs, 1H), 5.13 (s, 1H), 3.86 (s, 1H), 2.56 (t, 1H, J = 9.0 Hz), 2.22−2.14 (m, 1H), 2.08−0.84 (m, 23H), 2.05 (s, 3H), 1.01 (d, 3H, J = 6.6 Hz), 0.95 (d, 3H, J = 6.4 Hz), 0.94 (s, 3H), 0.51 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 209.8, 168.1, 74.0, 64.1, 58.8, 57.0, 44.5, 40.1, 39.4, 37.6, 35.8, 35.1, 31.8, 30.9, 30.6, 30.3, 26.5, 26.3, 25.1, 24.6, 24.0, 23.1, 21.3, 18.7, 18.4, 13.7. IR (film): 2928, 2865, 2620, 1734, 1703, 1378, 1354, 1224, 1154, 1019 cm^{-1}. HRMS-ESI m/z 418.3312 ([M − Cl]$^+$, $C_{26}H_{44}NO_3$ requires 418.3316). Anal. ($C_{26}H_{44}ClNO_3 + {}^1/_4H_2O$) C, H, N.

Pregn-4-ene-3,20-dione, Cyclic 20-(Ethylene Acetal) (29). Progesterone (3.46 g, 11.0 mmol) and ethylene glycol (110 mL, 1.98 mol, 180 equiv) were added to a 250 mL RBF. Activated powdered 4 Å molecular sieves (1.98 g) were added, followed by PTSA (2.09 g, 11.0 mmol, 1.00 equiv), and the reaction was stirred at room temperature for 5 d. Ether and saturated sodium bicarbonate were added. The aqueous phase was extracted with ether. The organic layers were combined and washed again with saturated sodium bicarbonate. The organic phase was separated, magnesium sulfate was added to the point of free-flowing, and the solution was stirred at room temperature overnight. The solution was filtered and concentrated, and the resulting white solid was loaded in a minimum amount of CH_2Cl_2 onto a 120 g silica column and eluted with 0−30% EtOAc in hexanes over 45 min. The desired C-20 ketal was recovered as 3.20 g (81%) white solid; R_f = 0.38 (1:1 EtOAc/hexanes, PMA stain). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.72 (s, 1 H), 4.03−3.84 (m, 4 H), 2.48−2.22 (m, 4 H), 2.15−1.98 (m, 2 H), 1.88−1.37 (m, 9 H), 1.29 (s, 3 H), 1.24−1.12 (m, 2 H), 1.18 (s, 3 H), 1.09−0.88 (m, 3 H), 0.81 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.9, 171.8, 124.0, 112.0, 65.4, 63.4, 58.3, 55.9, 53.9, 42.0, 39.4, 38.8, 35.9, 35.3, 34.2, 33.1, 32.1, 24.8, 23.9, 23.1, 21.0, 17.6, 13.1. IR (solid): 2937, 2882, 1668, 1621, 1437, 1372, 1228, 1051, 1040, 862, cm^{-1}. HRMS-ESI m/z 359.2577 ([M + H]$^+$, $C_{23}H_{35}O_3$ requires 359.2581).

Pregn-4-ene-3,20-dione, Cyclic 20-(Ethylene Acetal), 3-Oxime (30/31). Hydroxylamine HCl (2.78 g, 40.0 mmol, 4.00 equiv) was

added to a 100 mL oven-dried RBF with 15 mL of anhydrous CH_2Cl_2. Triethylamine (6.97 mL, 50.0 mmol, 5.00 equiv) was added, and the mixture was stirred for 45 min. Compound **29** (3.58 g, 10.0 mmol) was dissolved in 20 mL of anhydrous CH_2Cl_2 and added quickly dropwise to the reaction mixture. The reaction was stirred for 24 h at room temp. The solution was quenched with the addition of water. The organic layer was washed with water. The aqueous washes were combined and extracted with CH_2Cl_2. The organic layers were combined, dried, filtered, and concentrated with 10 g silica. The silica cake was eluted with a 0−25% EtOAc in hexanes gradient over 60 min on a 120 g silica column. The main products were recovered as 2.23 g (60%) *E* oxime and 1.33 g (36%) *Z* oxime, both as white solids. *E* isomer (**30**): R_f = 0.48 (1:1 EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (bs, 1H), 5.77 (s, 1H), 4.03−3.84 (m, 4H), 3.08−3.02 (m, 1H), 2.35−0.77 (m, 19H), 1.30 (s, 3H), 1.06 (s, 3H), 0.80 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.3, 156.0, 117.3, 112.1, 65.4, 63.4, 58.4, 56.1, 53.9, 42.0, 39.6, 38.1, 35.5, 34.8, 32.7, 32.3, 24.8, 24.0, 23.1, 21.3, 18.9, 18.0, 13.1. IR (solid): 3434, 2934, 2885, 1626, 1435, 1219, 1163, 1053 cm^{-1}. HRMS-ESI *m*/*z* 374.2677 ([M + H]$^+$, $C_{23}H_{36}NO_3$ requires 374.2690). *Z* isomer (**31**): R_f = 0.38 (1:1 EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (bs, 1H), 6.47 (d, 1H, *J* = 1.2 Hz), 4.03−3.84 (m, 4H), 2.40−0.78 (m, 20H), 1.30 (s, 3H), 1.10 (s, 3H), 0.80 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.0, 154.2, 112.1, 110.3, 65.4, 63.4, 58.3, 56.0, 54.1, 42.1, 39.5, 39.1, 36.4, 35.4, 33.2, 32.6, 24.9, 24.8, 23.9, 23.1, 21.2, 18.3, 13.2. IR (solid): 3403, 2936, 2870, 1626, 1434, 1218, 1150, 1052 cm^{-1}. HRMS-ESI *m*/*z* 374.2677 ([M + H]$^+$, $C_{23}H_{36}NO_3$ requires 374.2690).

Pregn-4-ene-3,20-dione, Cyclic 20-(Ethylene Acetal), 3-*O*-(*N*-Fmoc-L-valine)-*E*-oxime (32). Prepared according to the method described for compound **4b**. Clear oil that foamed on drying (99% yield); R_f = 0.54 (1:1 EtOAc/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.77 (d, 2H, *J* = 7.8 Hz), 7.61 (dd, 2H, *J* = 7.2, 3.0 Hz), 7.42−7.39 (m, 2H), 7.32 (t, 2H, *J* = 7.6 Hz), 5.98 (s, 1H), 5.46 (d, 1H, *J* = 9.6 Hz), 4.44−4.40 (m, 3H), 4.24 (t, 1H, *J* = 7.2 Hz), 4.02−3.86 (m, 4H), 3.01 (d, 1H, *J* = 16.8), 2.37−0.81 (m, 20H), 1.30 (s, 3H), 1.08 (s, 3H), 1.02 (d, 3H, *J* = 7.2 Hz), 0.99 (d, 3H, *J* = 7.2 Hz), 0.80 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.1, 164.2, 162.4, 156.4, 144.1, 143.9, 127.9, 127.3, 125.3, 120.2, 115.9, 112.0, 67.3, 65.4, 63.4, 58.4, 58.3, 56.0, 53.7, 47.3, 42.0, 39.5, 38.3, 35.3, 34.4, 33.1, 32.1, 31.9, 24.7, 23.9, 23.1, 21.2, 21.0, 19.1, 18.0, 17.8, 13.1. IR (film): 3347, 2937, 2880, 1756, 1718, 1513, 1374, 1339, 1239, 911, 710 cm^{-1}. HRMS-ESI *m*/*z* 695.4059 ([M + H]$^+$, $C_{43}H_{55}N_2O_6$ requires 695.4055).

Pregn-4-ene-3,20-dione, 3-*O*-(*N*-Fmoc-L-valine)-*E*-oxime (33). Compound **32** (0.265 g, 0.381 mmol) was dissolved in 15 mL of acetone and 0.0164 g (0.0953 mmol, 0.250 equiv) PTSA was added. The reaction was stirred at room temperature for 2 h then heated to 40 °C for 1 h. Ethyl acetate was added, and the reaction was concentrated to remove acetone. Additional EtOAc was added and the organic layer was washed with water (2 × 10 mL). The aqueous layers were combined and extracted with EtOAc. The organic layers were combined, washed with brine, dried, filtered, and prepared as a silica cake. The cake was eluted on a 12 g silica column with a 0−35% EtOAc in hexanes gradient over 45 min. The main product was recovered as 0.245 g (99%) waxy off-white solid; R_f = 0.52 (1:1 EtOAc/hexanes, PMA). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.77 (d, 2H, *J* = 7.8 Hz), 7.61 (dd, 2H, *J* = 7.2, 2.4 Hz), 7.42−7.39 (m, 2H), 7.32 (t, 2H, *J* = 7.2 Hz), 6.00 (s, 1H), 5.46 (d, 2H, *J* = 9.6 Hz), 4.40−4.40 (m, 3H), 4.24 (t, 1H, *J* = 7.2 Hz), 3.03 (d, 1H, *J* = 17.4 Hz), 2.53 (t, 1H, *J* = 9.6 Hz), 2.37−0.85 (m, 19H), 2.12 (s, 3H), 1.08 (s, 3H), 1.02 (d, 3H, *J* = 7.2 Hz), 0.99 (d, 3H, *J* = 6.6 Hz), 0.65 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 209.6, 170.1, 164.1, 161.8, 156.4, 144.1, 143.9, 141.5, 127.9, 127.3, 125.3, 120.2, 116.2, 67.4, 63.7, 58.4, 56.3, 53.6, 47.4, 44.1, 38.9, 38.3, 35.8, 34.4, 32.9, 32.1, 31.9, 31.7, 24.6, 23.0, 21.4, 21.0, 19.1, 18.1, 17.8, 13.5. HRMS-ESI *m*/*z* 651.3799 ([M + H]$^+$, $C_{41}H_{51}N_2O_5$ requires 651.3793).

Pregn-4-ene-3,20-dione, 3-*O*-(L-valine)-*E*-oxime Hydrochloride (34). Oxime compound **33** (0.260 g, 0.400 mmol) was added to an oven-dried 50 mL RBF and the flask was evacuated and inert gas flushed. Anhydrous ACN (20 mL) was added, and the clear colorless solution was chilled to 0 °C. Freshly distilled piperidine (0.395 mL, 4.00 mmol, 10.0 equiv) was added, and the solution was stirred and allowed to gradually equilibrate to room temperature over 30 min. The reaction was concentrated with added toluene to give a clear oil. The oil was loaded neat with minimum CH_2Cl_2 rinse onto a 12 g silica column and eluted with 0−80% EtOAc in hexanes over 40 min. Main product containing fractions were combined and concentrated by rotary evaporation at 10 °C. After being brought to complete dryness and redissolved in 10 mL of EtOAc, the sample was concentrated and dried under high vacuum while being chilled in an ice bath. Anhydrous ether was added, and the clear colorless solution was allowed to cool to 0 °C. HCl ether solution (0.195 mL, 2.0 M. 1.0 equiv) was added dropwise to the rapidly stirring solution. The mixture was stirred for 15 min at 0 °C, and the resulting white precipitate was filtered and washed with ice chilled anhydrous ether. White solid (67% yield, two steps); mp 144−146 °C; R_f = 0.25 (1:1 EtOAc/hexanes, PMA). ^{1}H NMR (400 MHz, DMSO) δ 8.71 (bs, 2H), 5.90 (s, 1H), 4.00 (bs, 1H), 3.53 (bs, 1H), 3.03 (d, 1H, *J* = 16.8 Hz), 2.56 (t, 1H, *J* = 9.0 Hz), 2.40−0.79 (m, 20H), 2.06 (s, 3H), 1.05 (s, 3H), 1.02 (d, 3H, *J* = 6.8 Hz), 0.96 (d, 3H, *J* = 6.8 Hz), 0.56 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 209.6, 166.5, 164.8, 162.5, 115.9, 63.7, 58.4, 56.3, 53.6, 44.1, 38.9, 38.3, 35.8, 34.5, 33.0, 32.2, 31.8, 30.3, 24.6, 23.0, 21.5, 19.2, 18.4, 17.9, 15.3, 13.5. IR (solid): 2933, 2874, 2648, 1760, 1699, 1627, 1377, 1358, 1185, 847 cm^{-1}. HRMS-ESI *m*/*z* 429.3109 ([M − Cl]$^+$, $C_{26}H_{41}N_2O_3$ requires 429.3112). Anal. ($C_{26}H_{41}ClN_2O_3 + {}^1/_2H_2O$) C, H, N.

The following compounds were prepared according to the methods developed for the *E*-oxime progesterone derivative **34**:

Pregn-4-ene-3,20-dione, Cyclic 20-(Ethylene Acetal), 3-*O*-(*N*-Fmoc-L-valine)-*Z*-oxime (35). White foam (99% yield); R_f = 0.50 (1:1 EtOAc/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.77 (d, 2H, *J* = 7.8 Hz), 7.62−7.60 (m, 2H), 7.43−7.36 (m, 2H), 7.32 (t, 2H, *J* = 7.2 Hz), 6.33 (s, 1H), 5.45 (d, 1H, *J* = 9.6 Hz), 4.44 (dd, 1H, *J* = 9.0, 4.8 Hz), 4.40 (d, 2H, *J* = 7.2 Hz), 4.24 (t, 1H, *J* = 7.2 Hz), 4.03−3.84 (m, 4H), 2.60−0.74 (m, 21H), 1.30 (s, 3H), 1.12 (s, 3H), 1.04 (d, 3H, *J* = 6.6 Hz), 1.00 (d, 3H, *J* = 7.2 Hz), 0.80 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.2, 165.7, 161.4, 156.4, 144.1, 144.0, 141.5, 127.9, 127.3, 125.3, 120.2, 112.0, 110.8, 67.3, 65.4, 63.4, 58.4, 58.3, 56.0, 54.0, 47.4, 42.1, 39.5, 39.4, 35.7, 35.3, 34.2, 33.5, 32.6, 31.9, 25.8, 23.9, 23.1, 21.1, 19.3, 18.0, 17.9, 13.1. IR (solid): 3318, 2935, 2876, 1716, 1468, 1371, 1309, 1236, 1042, 862, 739 cm^{-1}. HRMS-ESI *m*/*z* 695.4054 ([M + H]$^+$, $C_{43}H_{55}N_2O_6$ requires 695.4066).

Pregn-4-ene-3,20-dione, 3-*O*-(*N*-Fmoc-L-valine)-*Z*-oxime (36). White foam (89% yield); R_f = 0.40 (1:1 EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, 2H, *J* = 7.6 Hz), 7.61 (dd, 2H, *J* = 7.2, 2.4 Hz), 7.40 (t, 2H, *J* = 7.6 Hz), 7.32 (t, 2H, *J* = 7.6 Hz), 6.35 (s, 1H), 5.47 (d, 1H, *J* = 8.8 Hz), 4.44 (dd, 1H, *J* = 9.2, 4.8 Hz), 4.40 (d, 2H, *J* = 6.8 Hz), 4.24 (t, 1H, *J* = 7.2 Hz), 2.62−0.86 (m, 21H), 2.12 (s, 3H), 1.11 (s, 3H), 1.04 (d, 3H, *J* = 6.8 Hz), 1.00 (d, 3H, *J* = 7.2 Hz), 0.65 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 209.6, 170.2, 165.1, 161.3, 156.4, 144.1, 143.9, 141.5, 127.9, 127.3, 125.3, 120.2, 110.9, 67.3, 63.7, 58.4, 56.2, 53.8, 47.3, 44.1, 39.3, 38.8, 35.7 (2C), 33.3, 32.5, 31.9, 31.7, 24.7, 24.5, 23.0, 21.2, 19.3, 18.0, 17.9, 13.5. IR (solid): 3327, 2935, 1699, 1449, 1371, 1355, 1235, 1032, 859, 760, 740 cm^{-1}. HRMS-ESI *m*/*z* 651.3793 ([M + H]$^+$, $C_{41}H_{51}N_2O_5$ requires 651.3793).

Pregn-4-ene-3,20-dione, 3-*O*-(L-valine)-*Z*-oxime Hydrochloride (37). White solid (61% yield, two steps); mp 140−142 °C. ^{1}H NMR (400 MHz, DMSO) δ 8.74 (bs, 3H), 6.50 (s, 1H), 3.98 (s, 1H), 3.37 (s, 1H), 2.56 (t, 1H, *J* = 8.8 Hz), 2.48−0.82 (m, 20H), 2.06 (s, 3H), 1.09 (s, 3H), 1.01 (d, 3H, *J* = 7.2 Hz), 0.98

(d, 3H, J = 6.4 Hz), 0.56 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.6, 167.0, 166.0, 162.0, 111.3, 63.7, 58.2, 56.2, 53.8, 44.1, 39.3, 38.9, 35.7 (2C), 33.3, 32.5, 31.7, 30.3, 24.7, 24.6, 23.0, 21.3, 19.1, 18.5, 18.1, 13.6. IR (solid): 2935, 1756, 1698, 1620, 1379, 1356, 1185, 852 cm^{-1}. HRMS-ESI m/z 429.3108 ([M − Cl]$^+$, $C_{26}H_{41}N_2O_3$ requires 429.3112). Anal. ($C_{26}H_{41}ClN_2O_3$ + $^1/_2H_2O$) C, H, N.

Biological Assay Methods. MTT Assay. Primary cortical cells (NeuroPure E18 primary rat cortical cells; Genlantis) were seeded in multiwell plates and cultured for 8−10 days. Cells were then pretreated with various concentrations of a different PROG analogue (0.1, 1, 5, 10, 20, 40, and 80 μM) for 24 h. Cells were next exposed to glutamate (0.5 μM) for the following 24 h. Cytotoxicity was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale-yellow MTT. This reaction forms dark-blue formazan crystals, which are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. Solubilization of the cells results in the liberation of the crystals, which are then also solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. Concentrations were determined by photometric analysis.

Cerebral Edema Assay. Surgery. Contusions of the mediofrontal cortex (MFC) were created with a pneumatic impactor device. Animals were anesthetized using isoflurane (5% induction, 2% maintenance, 700 mm N_2O, 300 mm O_2), and mounted in a stereotaxic device with the head in a horizontal position. The body core temperature was maintained with a homeothermic heating blanket system. Using a SurgiVet (model V3304) pulse oximeter, blood SpO_2 was monitored and maintained at levels ≥90%. Under aseptic conditions, a midline incision was made in the scalp and the fascia retracted to expose the cranium. A centered, bilateral craniotomy was made 3 mm anterior to bregma using a 6 mm diameter trephan. After the removal of the bone, the tip of the impactor was moved to AP 3.0, ML 0.0, checked for adequate clearance, retracted to its elevated position, and lowered 3.5 mm DV. The contusion was then made at a velocity of 2.25 m/s with a brain contact time of 0.5 s. Following this procedure, the wound cavity was thoroughly cleaned and all bleeding stopped before the fascia and scalp were sutured closed.

Progestin Preparation and Administration. All experimental treatments by injection (progesterone and analogues) were made in stock solutions using 2-hydroxypropyl-β-cyclodextrin (HBC; 22.5% w/v solution in H_2O) as the solvent. The HBC vehicle allows progesterone and analogues to be dissolved in a nontoxic, aqueous solution, which can be administered safely by a variety of routes. The initial dose of progesterone or analogue given at 1 h postinjury was delivered IP for rapid absorption followed by SC injection at 6 h postinjury.

Edema Measure. At 24 h postinjury, animals were given an IP overdose of pentobarbital (75 mg/kg). The pericontusional tissue samples from each area were assayed for water content as follows: samples were placed into preweighed containers, capped, and then immediately weighed to the nearest 0.0001 g. The containers were then uncapped and placed in a vacuum oven and dried at 60 °C, 0.3 atm for 24 h. The containers were then recapped and reweighed to obtain the dry and wet-weight percentages. A "% mean difference" value could then be calculated based on the relative edema difference between injured and noninjured tissue samples for a given animal. The percent water content of each sample was calculated as [(wet wt − dry wt)/wet weight] × 100. The percent difference in water content of the area surrounding the injury was compared to an area distal to the injury [(contusion area − distal area/distal area) × 100].

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Supporting Information Available: Table of elemental analysis data for compounds **6a**−**6e**, **11**, **16**, **19**, **25**, **28**, **34**, and **37**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Appendix 2: Sayeed, I., MacNevin, C.J., Atif, F., Liotta, D.C., Stein, D.G., Development and screening of water soluble analogs of progesterone: Potential for an innovative, safe and effective approach to acute traumatic brain injury treatment. Society for Neuroscience Annual Meeting, Oct 19, 2009, poster 335.4/K24.

Program#/Poster#: 335.4/K24

Title: Development and screening of water soluble analogues of progesterone: Potential for an innovative, safe and effective approach to acute traumatic brain injury treatment

Location: South Hall A

Presentation Time: Monday, Oct 19, 2009, 11:00 AM -12:00 PM

Authors: ***I. SAYEED**[1], C. J. MACNEVIN[2], F. ATIF[1], D. C. LIOTTA[2], D. G. STEIN[1];
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Abstract: **Objective: Pre-clinical and clinical research has shown that the hormone progesterone (PROG) is neuroprotective when given in the first hours after a traumatic brain injury (TBI). However, the use of PROG as a therapeutic agent suffers from a number of practical limitations, including its poor solubility and the instability of its formulation. Therefore, our primary objective was to develop and screen a new PROG pro-drug (PD) that is more potent, stable and easily administered by direct injection under emergency conditions. Methods: The strategy towards the preparation of an effective PROG PD was to ultimately achieve bioequivalence to PROG. Several chemically novel analogues of PROG were synthesized and screened for solubility, pharmacokinetics (PK), and neuroprotective efficacy in an in vitro model of glutamate-induced excitotoxicity in primary cortical neurons. The number of cells surviving glutamate challenge was the end-point measurement of neuroprotection. The analogues which successfully passed through cell-culture screening were then tested in vivo for their edema-reducing effects measured 24h after bilateral cortical contusion injury in adult male rats. Results: Based on solubility data we were able to identify two potent candidates: P1-185 (13 mg/ml) and P1-186 (15.6 mg/ml). Both showed much better solubility in water than PROG (<0.05 mg/ml). PK studies revealed that the oxime based compound P1-186 generated PROG in vivo when given IV at 10 mg/kg. Maximum serum levels of 100 ng/ml were attained over the course of 12h. *In vitro* data demonstrated that both analogues were more effective in reducing glutamate-induced neuronal loss compared to PROG at a similar (5µM) dose. Furthermore, P1-186 reduced edema as effectively as PROG in**

adult rats with TBI. Conclusions: The use of a more highly water soluble PD of PROG may be useful for delivering PROG to brain-injured subjects under emergency conditions and achieving neuroprotective effects.

Disclosures: **I. Sayeed**, Pending patent application entitled "Steroid Analogues for Neuroprotection.", E. Ownership Interest (stock, stock options, patent or other intellectual property); **C.J. MacNevin**, Pending patent application entitled "Steroid Analogues for Neuroprotection.", E. Ownership Interest (stock, stock options, patent or other intellectual property); **F. Atif**, None; **D.C. Liotta**, Pending patent application entitled "Steroid Analogues for Neuroprotection.", E. Ownership Interest (stock, stock options, patent or other intellectual property); **D.G. Stein**, Entitled to royalty payment from BHR Pharmaceuticals related to research on progesterone and brain injury. His future financial interests may be affected by the outcome of this research., E. Ownership Interest (stock, stock options, patent or other intellectual property).

Keyword(s): Progesterone

Traumatic Brain Injury

Drug development

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[Authors]. [Abstract Title]. Program No. XXX.XX. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online.

Appendix 3: Sayeed I, MacNevin CJ, Atif F, Nachus MG, Liotta DC, Stein DG. Development and screening of water soluble analogues of progesterone: Potential for an innovative, safe and effective innovative approach to acute traumatic brain injury treatment. Military Health Research Forum, Kansas City MO, August 31-September 3, 2009.

Development and screening of water soluble analogues of progesterone: Potential for an innovative, safe and effective innovative approach to acute traumatic brain injury treatment

Iqbal Sayeed[a], Christopher J. MacNevin[b], Fahim Atif[a], Michael G. Nachus[b], Dennis C. Liotta[b], Donald G. Stein[a]

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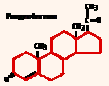
Introduction

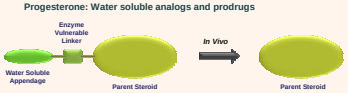
- Progesterone's (PROG) safety profile in clinical trial has been excellent and proof-of-principle for efficacy has been demonstrated in two independent Phase II trials. A Phase III, NIH-sponsored, multi-center national trial for PROG in TBI will begin in October 2009.
- Although successful, the use of PROG in the Phase II trial had several requirements that would make treatment in far-forward battlefield conditions impractical. Natural PROG presents several challenges for field use:
  - Poor solubility – formulated in the hospital for each patient
  - Narrow window of therapeutic opportunity
  - Poor oral availability and short half-life



- Addressing obstacles that limit the usefulness of the natural hormone in battlefield conditions will help obtain the best possible clinical outcome.
- TBI produces a complex succession of molecular events in addition to the immediate loss of nervous tissue caused by concussions, contusions and ballistic injuries. The "brain injury cascade" unfolds over days, weeks and even months after the initial trauma.

Goals

- Screen and develop PROG analog that can be easily, rapidly and effectively administered on-site in an emergency battlefield situation for TBI.



Materials & Methods

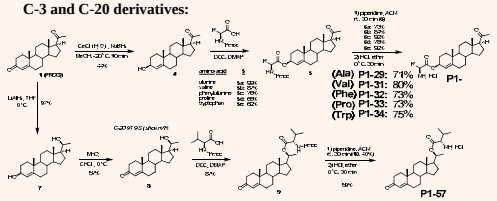
- *In vitro* injury model
  - Glutamate-induced toxicity
  - Primary cortical cells were seeded in multi-well plates
  - Cultured for 8-10 days before any treatment
  - Cell death assays**:**
    - Lactate dehydrogenase (LDH) Release
    - MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
- Rat model of TBI
  - Male Sprague-Dawley rats, 250-260 g, 60 days old.
  - Controlled cortical impact (CCI) of medial frontal cortex analogous to human prefrontal cortex; velocity = 2.25 m/s, depth = 2 mm; duration = 150 ms
- Treatments
  - Injections of PROG and PROG-PD in HBC (8 mg/kg for TBI)
  - Administered i.p. at 1 h and s.c. at 6 h post-TBI
  - Brain samples extracted at 24 h
- Edema Assay
  - Percent water content
  - Calculated as [(wet wt - dry wt)/wet weight] X 100

Results

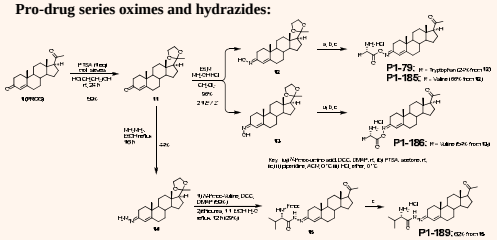
Synthesis of Compounds

- Initial approach: exploring regioselectivity of substitution and degree of saturation factor
- Different amino acids were used as solubilizing groups.

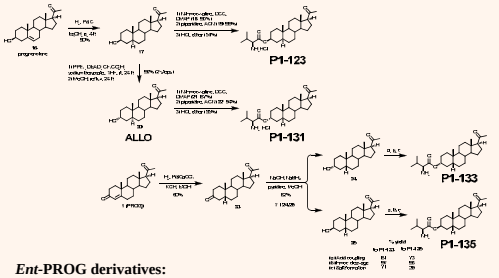
C-3 and C-20 derivatives:



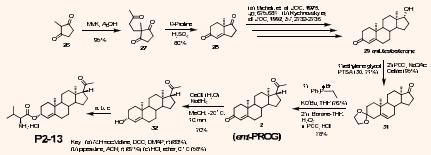
Pro-drug series oximes and hydrazides:

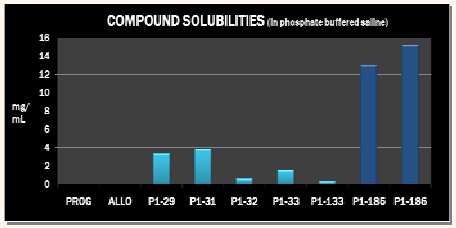


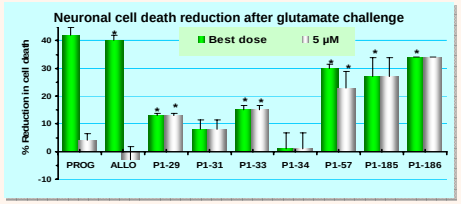
ALLO derivative series:

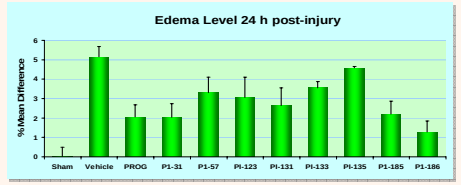


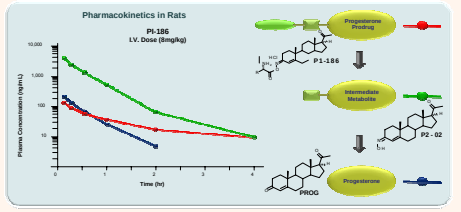
***Ent*-PROG derivatives:**











Summary

- The use of an amino acid tether significantly improves solubility of PROG and related steroidal compounds.
- Of prepared compounds screened to date, oxime derivatives, C-3 reduced analogues, and ALLO-derived compounds have shown the best efficacy in reducing cerebral edema.
- Oxime compound P1-186 has been shown to readily convert to its parent oxime and generates PROG in vivo when dosed i.v.

Support for this project was provided by DOD Grant W81XWH-08-1-0184 and the Emory Institute for Drug Discovery

Appendix 4: Patent application: Steroid analogs for neuroprotection, US Provisional Patent Application Nos. 61/032,315, 61/031,629, and 61/031,567.

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January 30, 2009

VIA U.S. MAIL

Cory Acuff, Ph.D.
Office of Technology Transfer
Emory University
1599 Clifton Road, NE,
Fourth Floor, Mail Stop 1599-001-1AZ
Atlanta, GA 30322

Re: U.S. Provisional Patent Application Nos. 61/032,315, 61/031,629, and 61/031,567
entitled "Steroid Analogues for Neuroprotection"
K&S File No. 18085.105378 US
Emory Ref. No. 08042

Dear Dr. Acuff:

As you know the above-identified provisional patent applications were filed in the U.S. Patent and Trademark Office on February 28, 2008, February 26, 2008, and February 26, 2008 respectivley. This application must be converted to a normal U.S. application and an international PCT application on or before **February 26, 2009**.

Please sign the attached sheet and return it to our office with your instructions on whether to file this application as a U.S. normal and PCT application by **January 26, 2009**.

If you desire to proceed with this application, corrections or additional data to the specification should be provided to us by **January 26, 2009**, so that we can incorporate the changes and prepare the application for filing in the respective offices.

In addition, if you desire to file this application in the any non-PCT states named on the attached pages, we will require these instructions by **January 26, 2009**, in order to allow time for translation.

By copy of this letter to the inventors, we also enclose an assignment documents. All inventors should sign these documents and return the originals to our office.

If you have any questions do not hesitate to contact me.

Sincerely yours,

Sally Sexton

Sally Sexton
Patent Paralegal

cc: Dennis C. Liotta
Donald G. Stein
Christopher MacNevin
Iqbal Sayeed

____ We do/do not wish to convert U.S. provisional application nos. **61/032,315, 61/031,629, and 61/031,567** to a normal U.S. application.

____ We do/do not wish to convert U.S. provisional application no. **61/032,315, 61/031,629, and 61/031,567** to a PCT international application.

We understand that a decision not to convert this application will result in the lapse of this patent.

Date:_____ By:_____

Appendix 5: Pre-proposal, **"**Water soluble progesterone derivative in the far-forward treatment of TBI," (DM102619) submitted to DOD December 18, 2009. PI, Donald G. Stein, Ph.D

Defense Medical Research and Development Program Pre-Proposal Form - Part 1

1. Proposal Information

*PI Last Name: Stein
*PI First Name: Donald
Proposal Title: Water soluble progesterone derivative in the field-forward treatment of TBI
eReceipt Log No.: DM102619
DMRDP Project : FY10 Diagonosis and Treatment of Brain Injury
DMRDP Task: Far Forward Diagonosis and Treatment of TBI

2. **Specific Hypothesis/Aims (Problems to be Studied)** (Max 2,300 Characters including spaces)

Development of a safe and effective field-forward treatment to provide neuroprotection, enhance neuronal repair and prevent the secondary loss of brain cells after a traumatic brain injury (TBI) is an urgent military priority. The objective of our proposed *pre-clinical* project is to develop such a treatment and thereby to prevent cognitive, sensory and motor deficits after TBI. With funding from the Department of Defense (Concept Award W81XWH-08-1-0184) we recently succeeded in synthesizing several new, more hydrophilic progesterone (PROG) derivatives that can be directly injected and may be as effective in reducing TBI-induced cerebral edema as natural (lipid-soluble) progesterone (nPROG). Our objective now is to carry out detailed preclinical, pharmacokinetic and toxicokinetic studies on the selected PROG prodrug using a controlled impact injury to the frontal cortex in laboratory rats. We will determine best dose and window of opportunity for treatment compared to animals given nPROG. All treated animals will be compared to appropriate controls, based on morphological and functional outcomes that are used routinely in TBI research. **Hypothesis**: *A new PROG derivative that is a more soluble, potent, stable and effective field-forward treatment than nPROG will reduce neuronal loss and enhance cognitive, sensory and motor recovery after TBI.* **Military Relevance:** An effective neuroprotective agent easily administered on the battlefield would represent a major breakthrough for military medicine. This proposal is consistent with the "Dignified Treatment of Wounded Warriors Act," bipartisan legislation approved by the United States Senate Armed Services Committee on May 3, 2007. An amendment to this bill directs the Department of Defense to review recent findings regarding progesterone and the treatment of TBI. It also directs the Department of Defense to collaborate with other federal agencies, such as the NIH, regarding research and clinical trials related to TBI.

3. Scientific Rationale (Max 2,300 Characters including spaces)

Natural PROG has impressive neuroprotective properties but it also has limitations that make it problematic for use under highly variable or difficult environmental conditions where solubility and stability are at issue. This presents an opportunity to develop a novel molecule that maintains PROG's therapeutic properties while overcoming its liabilities, especially in emergency applications. We have synthesized several PROG analogues that can improve formulation and administration of nPROG in emergency situations (J Med Chem 8;52(19):6012-23). Patent

applications claiming composition of matter have been filed for several of the compounds. The process for creating the PROG prodrugs entailed attaching a water soluble appendage to PROG through a linker that was easily cleaved *in vivo.* Several of these compounds had a much higher level of solubility and showed neuroprotective effects. We have screened and selected the derivatives that are: (1) more stable for storage and readily administered under a range of field conditions; (2) more readily metabolized *in vivo* to give therapeutic levels of PROG; and (3) showed efficacy in preliminary assessments in rats. To advance to field testing the new formulations must be tested for safety and functional efficacy in whole animal models of TBI.

Our group has studied PROG as a safe and effective treatment for TBI in young and senescent, male and female, laboratory animals for more than 25 years. Although we have synthesized new PROG derivatives demonstrating the desired properties *in vivo,* we now need to determine if we can enhance neuroprotection and improve functional recovery in animal models of moderate to severe TBI. We need to determine how they will compare to PROG with respect to pharmacokinetics, dose, and duration of treatment. With more than 40 years of experience in the field of TBI research we propose that:

1. Our laboratory can provide expert pre-clinical evaluation of a potentially safe and effective treatment for TBI.
2. We have the facilities and skills to examine short- and long-term molecular, physiological and behavioral outcomes of TBI.
3. With our experience and facilities, we can perform these studies in a timely and cost-effective manner.

4. Approach/Methods (Max 2,300 Characters including spaces)

Briefly describe the experimental design, methods, and materials that are planned to accomplish the proposed research. For human studies, this should include a description of the size and characteristics of the subject population that will be employed.

We will test the selected PROG prodrug in rats with a controlled bilateral injury to the medial frontal cortex (MFC) to determine the extent of morphological and functional recovery. *Surgery:* We will create bilateral contusions of the MFC with a magnetic impactor device. In all experiments, the rats' group identity will be coded with regard to surgery and treatment to prevent experimenter bias. *Dose response and window of treatment:* To determine safety and efficacy, we will test three doses of the prodrug selected on the basis of *in vitro* evidence of neuroprotection and toxicity studies in primary cortical neurons. To determine window of treatment opportunity, we will use the best dose of prodrug to treat different groups of brain-injured rats at 1, 24, 48 and 72 hours after injury. *Behavioral testing*: The animals will be tested pre-injury to establish baseline performance on a number of different behavioral tests including: spontaneous motor behavior (a measure of habituation/ hyperactivity), grip strength, rotarod and tactile adhesive removal (measure of sensory neglect), then retested at specific times post-injury. The rats will also be tested for cognitive and spatial navigational performance. During behavioral testing the experimenters will be blinded to surgical and treatment conditions. *Histology*: Following behavioral testing brains will be processed for cryo-sectioning and histological evaluation. By examining neuronal and glial morphology we will determine the extent of neuroprotection provided by the prodrug treatments. We will also label and count microglia and astroglia to determine the effect of PROG prodrug treatment on post-injury reactive gliosis.

This work will be an important first step in advancing to a safe and effective field treatment for serious brain injuries occurring in a combat situation.